MECHANICAL STRESS INDUCES RESISTANCE AGAINST WILT DISEASE CAUSED BY *FUSARIUM OXYSPORUM* IN *ACACIA KOA*

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ABSTRACT

Acacia koa, an economically important timber tree in the Hawaiian Islands, is suffering from a devastating fungal wilt and dieback disease caused by *Fusarium oxysporum* f. sp. *koae*. Previous studies have shown that some environmental stresses, such as drought and wind, can enhance disease resistance in plants. In this research, the effects of a non-wounding mechanical stress on disease resistance were studied in *A. koa*. The specific objectives were (i) to identify actively transcribed genes through transcriptome analysis, (ii) to identify morphological, biochemical, and transcriptional changes induced by a non-wounding mechanical stress, and (iii) to determine the level of resistance in mechanically stressed *A. koa*. To identify genes related to disease resistance, 85,533 unigenes were assembled through Illumina sequencing and Trinity *de novo* software, and 47,038 unigenes were annotated based on search for sequence similarity with known proteins. From these sequences, 4,000 genes related to plant defense and growth were selected for a microarray analysis to determine if they were mechanically induced. For a non-wounding mechanical stress, *A. koa* seedlings were gently bent once in each of the four cardinal directions. The gene expression analyses showed upregulation of over 50 genes (> 2-fold) within 10-60 min following the treatment. They included genes for calcium, hormone, and MAPK signaling, and disease resistance. Genes for pathogenesis-related proteins and secondary metabolite biosynthesis were also upregulated within 6 h. For morphological and biochemical changes, plants were mechanically treated daily for 2-6 mo; the stressed plants had significantly reduced stem length, whereas they had significantly increased stem diameter, and anthocyanin and lignin contents. The disk diffusion assays and qRT-PCR analyses were used to determine the level of induced resistance. The results showed that the stressed plants had stronger antifungal activities and were 'primed' for defense with enhanced expression levels of defense-related genes following fungal inoculation. Consistently, the stressed plants had a significantly higher survival rate following a 100-day *F. oxysporum* inoculation trial. These results indicate that disease resistance can be induced and studied simply by providing the non-wounding mechanical stress; it may contribute towards identifying useful biomarkers for *A. koa* seedling selection of disease resistance.

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LIST OF ABBREVIATIONS

Acronym	Definition
4CL	*p*-coumarate:CoA ligase
ABA	Abscisic acid
AS	Anthranilate synthase
BAK1	Brassinosteroid insensitive 1-associated receptor kinase 1
Bgl	β-1,3,glucanase
C3H	*p*-coumarate 3-hydroxylase
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
CCoAOMT	Caffeoyl-CoA O-methyltransferase
CCR	Cinnamoyl CoA reductase
CDPK	Calcium-dependent protein kinase
CEBiP	Chitin-elicitor binding protein
CERK1	Chitin-elicitor receptor kinase 1
CHS	Chalcone synthase
CM	Chorismate mutase
CML	Calmodulin
CNGC	Cyclic nucleotide-gated channel
COMT	Caffeic acid 3-O-methyltransferase
CS	Chorismate synthase
DAMP	Damage-associated molecular patterns
DFR	Dihydroflavonol-4-reductase
DTI	DAMP-triggered immunity
ET	Ethylene
ETI	Effector-triggered immunity
F5H	Ferule 5-hydroxylase
HCT	Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase
HR	Hypersensitive reaction
ISR	Induced systemic resistance
JA	Jasmonic acid
MAMP	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MTI	MAMP-triggered immunity
NBS-LRR	Nucleotide-binding site leucine-rich repeat
NO	Nitric oxide
NOS	NO synthase/NO-associated protein 1
PAL	Pheylalanine ammonia-lyase
PRR	Pattern-recognition receptor
Rboh	Respiratory burst oxidase homolog
RING	RING finger protein
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
ZFP	Zinc finger protein

CHAPTER 1
INTRODUCTION

Acacia koa: a beautiful timber-wood tree on the Hawaiian Islands

1.1. Acacia koa

Figure 1. *Acacia koa* tree from the Aiea Loop Trail, Aiea, Hawai‘i.

Acacia koa A. Gray (koa) is an important leguminous tree endemic to the Hawaiian Islands (Fig. 1). It is a member of the legume family (*Fabaceae)* and the mimosa subfamily (*Mimosoideae*). The native *A. koa* forests are broadly distributed across all six major Hawaiian Islands, Hawai‘i, Moloka‘i, Maui, Lāna‘i, O‘ahu, and Kaua‘i (Wagner et al. 1999). *Acacia koa* is found in a broad range of habitats; it grows at elevations from near sea level to 2000 m, in mesic or wet forests with annual rainfall from 1850 to 5000 mm (Harrington et al. 1995; Anderson et al. 2002; Wilkinson and Elevitch 2003; Baker et al. 2009). It is a fast-growing tree, growing at the rate of ~1.5 m in height per year for the first five years under favorable conditions (Elevitch et al. 2006). It is the largest native tree of the Hawaiian Islands and can grow over 30 m, but some populations have a smaller and shrubbier form (Elevitch et al. 2006; Baker et al.

2009). *Acacia koa* serves as a vital resource for the Hawaiian Islands, providing benefits ecologically, culturally, and economically.

First, as a nitrogen-fixing legume, *A. koa* enhances soil fertility of forests and provides habitats for many native fauna and flora, including the endangered Hawaiian honeycreepers, such as ʻakiapōlāʻau (*Hemignathus munroi*) and ʻākepa (*Loxops coccinea;* Sakai 1988; Whitesell 1990; Elevitch et al. 2006; Baker et al. 2009). Many native plant species are associated with *A. koa*, including ʻōhiʻa lehua (*Metrosideros polymorpha*), one of the most important trees in Hawaiian forests along with *A. koa*. Other important understory trees associated with *A. koa* include naio (*Myoporum sandwicense*), ʻōlapa (*Cheirodendron trigynum*), kāwaʻu (*Ilex anomala*), kōlea (*Myrsine lessertiana*), kōpiko (*Psychotria spp*.), ʻiliahi (*Santalum* spp., sandalwood), olopua (*Nestegis sandwicensis*), and pilo (*Coprosma* spp.; Mueller-Dombois and Fosberg 1998; Wagner et al. 1999; Elevitch et al. 2006; Baker et al. 2009). Secondly, *A. koa* is culturally very important. The word "koa" means bravery or warrior in Hawaiian, and it provides a valuable wood to native Hawaiians. It was traditionally associated with royalty and was used to make outrigger canoes for fishing, racing, and voyaging, canoe paddles, spears, and more recently, surfboards and ʻukuleles (Abbott 1992; Krauss 1993; Elevitch et al. 2006; Baker et al. 2009). In addition, the beautiful texture, hardiness, and carving quality of *A. koa* timber, also referred to as Hawaiian mahogany, make it a highly priced commodity with a current market value of up to $125 per board foot (Baker et al. 2009). The wood color, varying from blond to dark red, and the grain figures, ranging from plain to curly (highly figured), are the most important determinants of the price; curly wood with a dark red color is the most highly valued (Baker et al. 2009). The wood is used for fine furniture, decorative items, musical instruments, and jewelry. The gross value of *A. koa* timber and the wood products produced is estimated to be in the range of $20-$30 million annually (Yanagida et al. 2004; Baker et al. 2009).

Over the past century, cattle ranching, grazing by wild animals, agriculture, land developments, and spread of invasive plant species have severely reduced *A. koa* forests (Whitesell 1990; Ewel and Bigelow 1996). However, in recent years, due to an increased awareness of the importance of *A. koa*, there has been greater discussion in reestablishing *A. koa* forest using methods of sustainable land use systems that maximize profitability (Newell and Buck 1996; Mitchell et al. 2005; Perkins et al. 2014). For example, many agencies, such as the United States Fish and Wildlife Service (USFWS), the Division of Forestry and Wildlife (DOFAW), and the n (NPS), have begun growing *A. koa* in mesic montane forests and

parklands on the island of Hawai‘i to protect forest birds, such as ‘akiapōlā‘au and ‘ākepa, as well as to develop sustainable *A. koa* forestry that will help landowners maintain the economic value of their lands (Mitchell et al. 2005).

1.2. Long-term goals of the *A. koa* improvement program

Long-term goals of the *A. koa* improvement program include improvement in disease resistance, wood quality, and plant architecture. Variations in wilt disease resistance, wood quality, tree architecture, and other traits have been observed among various *A. koa* populations, and these phenotypic differences appear to be heritable (Sun 1996; Brewbaker 1997; Sun et al. 1997; Daehler et al. 1999; Dudley et al. 2007*a* and 2007*b*). However, genetic improvement in *A. koa* is difficult because the tetraploidy and cross-pollinating nature of *A. koa* make the plant highly heterozygous and heterogeneous. Consequently, progenies of a single mother tree are genetically different and may not have the same traits as the mother tree, and it is difficult to grow a uniform population of *A. koa* trees with desired qualities. Therefore, it is necessary to develop methods to effectively select *A. koa* with desired properties, especially disease resistance, wood quality, and plant architecture.

1.2.1. Disease resistance

In spite of the growing interest in *A. koa* forests, restoration attempts are hampered due to a devastating vascular wilt and dieback disease caused by a fungal pathogen *Fusarium oxysporum* f. sp. *koae*. This invasion has been the major cause of decline of *A. koa* in the native Hawaiian forests in recent years (Gardner 1980, 1996; Anderson and Gardner 1998; Anderson et al. 2002; James 2005; Pejchar and Press 2006; Dudley et al. 2007*a*). *Fusarium oxysporum* is a soil-borne fungus that typically infects the host plants through the root system (MacHardy and Beckmann 1981). Upon entering the roots, mycelium advance intercellularly into the plant’s xylem vessels where it is then able to spread upwards. This mycelial invasion subsequently leads to clogging of vessels due to the physical obstruction by the mycelium and formation of host plant defense compounds, such as tyloses and gum (Fig. 2; Pietro et al. 2003). Once too many xylem vessels are clogged, the host dies due to the disruption of water movement (MacHardy and Beckmann 1981). *Fusarium oxysporum* can also survive an extended period of time in soil without any available host; as such controlling this hardy and ubiquitous pathogen has been difficult.

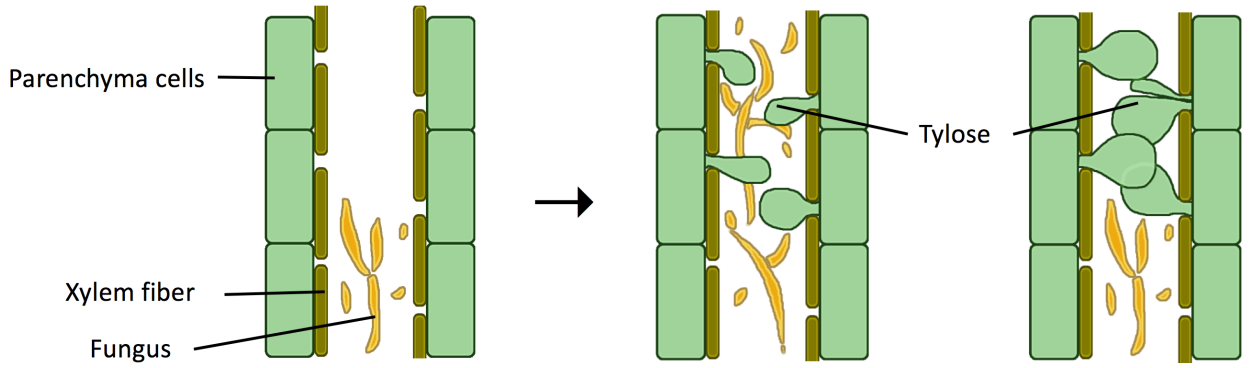

Figure 2. Clogging of xylem vessels by *Fusarium oxysporum.* The fungal pathogen invades the host plant intracellularly through xylem vessels. The vessels eventually are clogged due to the accumulation of mycelium and formation of host plant defense compounds, such as tyloses and gum, leading to drought-like symptoms.

Efforts have been made to breed *A. koa* for wilt resistance by selecting resistant families derived from single mother trees. In field trials performed by Shi and Brewbaker (2004), seedlings were exposed to naturally occurring levels of *F. oxysporum* infection to determine the long term survival rates. James et al. (2005) and Dudley et al. (2007*a* and 2007*b*) isolated virulent strains of *F. oxysporum* f. sp. *koae*, and they have been used to artificially inoculate the seedlings in 100-day greenhouse trials to determine the survival rate and select resistant families (Dudley et al. 2015). Field trials are currently being conducted to validate the results of the greenhouse trials. These results may be used to confirm if this screening method can be used to select resistant *A. koa* families prior to out-planting (Dudley et al. 2015). However, this process is both time-consuming and cost-ineffective because it can take up to several years (Rushanaedy et al. 2012). For more rapid selection, molecular approaches are also being developed to select for disease resistance. Rushanaedy et al. (2012) observed that expression of chitinase genes *Akchit1a* and *Akchit1b* was higher in resistant koa families than in susceptible ones following inoculation by virulent strains of *F. oxysporum*. Chitinases are important defense enzymes that break down chitin in fungal cell wall infecting plants (Punja and Zhang 1993; Sharma et al. 2011), and those differentially expressed chitinases have potentials as markers for fast screening of resistant *A. koa*.

1.2.2. Wood quality

Important wood properties of *A koa* for selection include the grain figures and color (Fig. 3). These are influenced by environmental factors, such as geographic location, to a large extent (Loudat and Kanter 1996; Dudley and Yamasaki 2000). It appears that variations of the

grain figure also have a genetic basis; some *A. koa* have the most valued curly grain figure systemically throughout the entire trees, while others do not (Dudley 2007). Therefore, there is a potential for genetic improvement. The formation of the heartwood color is not well understood (Wilton et al. 2015), and the evaluation of clonal *A. koa* trees from different sites may help determine genetic and environmental factors affecting the wood color. Unfortunately, no definitive selection strategy is currently available for wood qualities in *A. koa*.

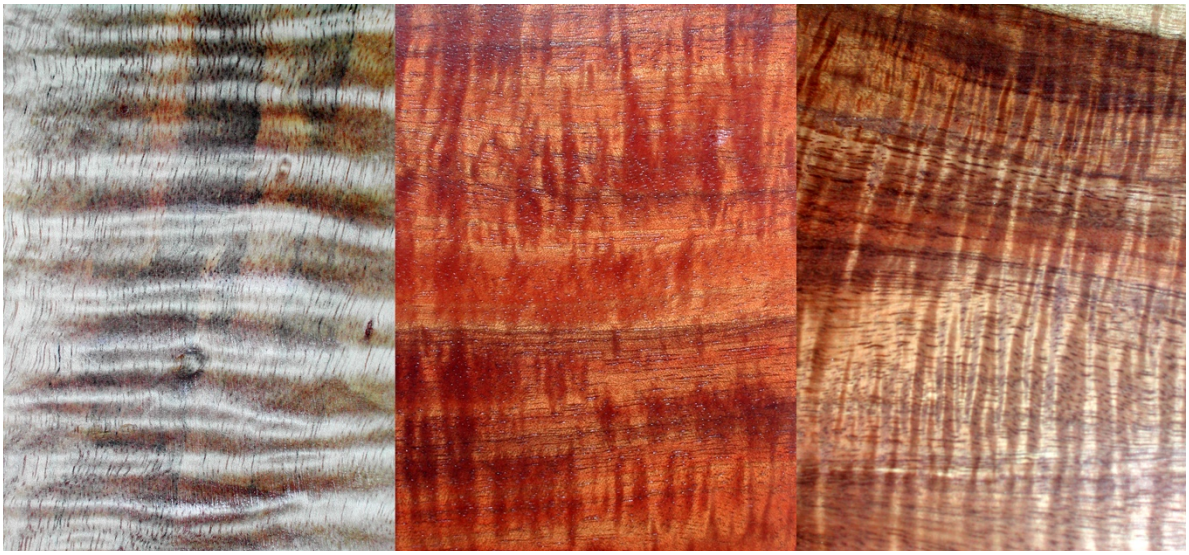

Figure 3. Different variations in color and grain figures of *Acacia koa*. Left to right: dark color banding, dark red color with curl, and brown color with curl. Picture courtesy of Nick Dudley and Tyler Jones.

1.2.3. Plant architecture

For lumber production, a long straight single trunk is ideal. However, the architecture of *A. koa* trees is highly variable; some trees form an upright, single trunk, while others have sprawling branches near the ground or multiple trunks (Fig. 4; Elevitch et al. 2006). Poor stem structure (*e.g.* branched, multi-trunked) is a major problem in *A. koa* forestry; the study by Scowcroft et al. (2010) showed that 50-80% of the trees in the tested plantations were not suitable as potential wood trees to produce merchantable timber due to their poor trunk structure. Although the trait of tree architecture appears heritable, the selection for superior tree form has not been successful due to the heterozygous and heterogeneous nature of *A. koa* and other environmental factors influencing this trait. For example, Daehler et al. (1999) reported that more than 50% of the progeny of mother trees with superior trunk architecture had major forks in the trunk close to the ground. Thus, poor tree form is a problem that reduces wood production by over 30%, even at *A. koa* plantations where the planting stocks came from

several mature trees of superior size and upright trunk (Skolmen et al. 1991; Scowcroft et al. 2010). Further research to determine the causes of poor stem form in *A. koa* plantations is necessary to improve the architecture of the plant.

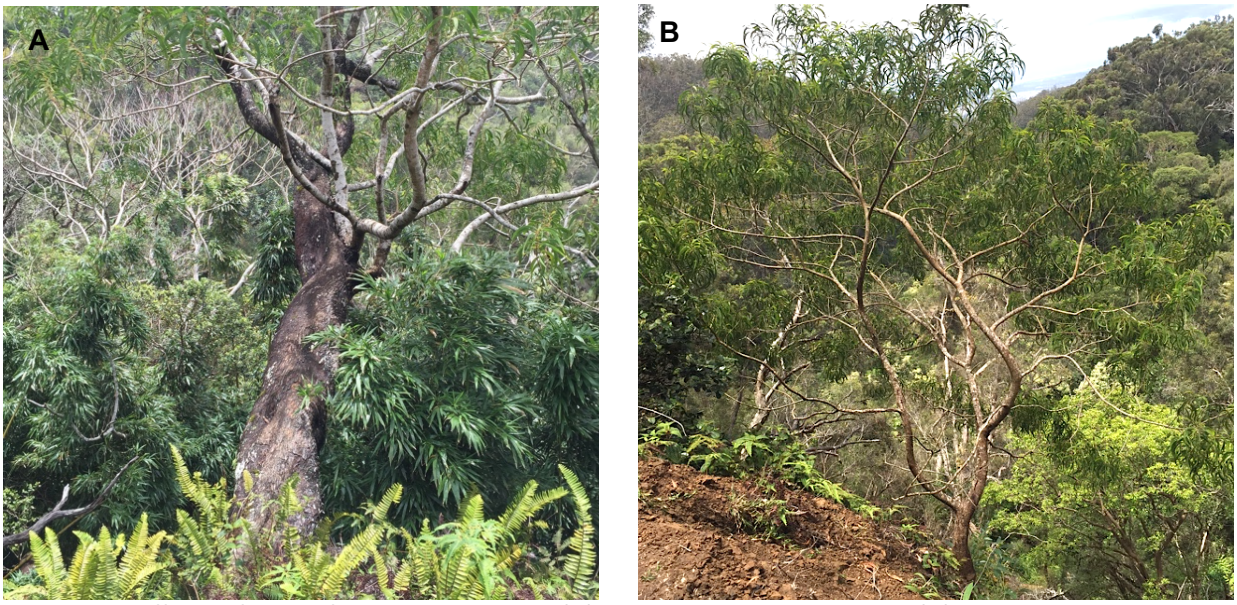

Figure 4. Different forms of *Acacia koa* trees: (A) a tree with a single trunk; (B) a branchy tree without a central trunk.

1.3. Hypotheses and Specific Objectives

This project will address the first issue, namely disease resistance against *F. oxysporum* f. sp. *koae.* Although potential selection methods have been currently developed, mechanisms underlying disease resistance in *A. koa* are not well-studied, and understanding of such mechanisms is necessary to facilitate growing resistant *A. koa* trees. Although some studies have shown that biotic and environmental stress responses can crosstalk with each other to produce antagonistic effects, other previous studies have shown that various environmental stresses, such as drought, cold, and wind, can induce disease resistance in plants. Based on these studies, it was hypothesized:

1. Genes involved in disease resistance will be induced by non-wounding mechanical stimuli
2. Mechanically treated *A. koa* plants will have increased disease resistance.

The goal of this study was to understand how mechanical stimuli affect disease resistance in *A. koa* in order to protect the forests from *F. oxysporum*. In this research, a 'non-wounding mechanical stress' is defined as a gentle bending movement of a stem from its

position at 90º with the ground level to a position at approximately 60º with the ground level in each of all the four cardinal directions. A 'mechanically stressed' plant is a plant that undergoes such movement whereas a 'unstressed' plant is a plant without such movement. If the hypotheses are correct, mechanically stressed *A. koa* may be used as a model to study disease resistance, and it can be used to elucidate defense mechanisms and to identify key molecules that influence disease resistance. Those molecules may be useful as markers to select for resistance or as diagnostic markers to determine if plants are infected at an early stage. The specific objectives of this research are as follows:

1. To identify actively transcribed genes in *A. koa* through transcriptome analysis.
2. To identify morphological, biochemical, and transcriptional changes in response to a non-wounding mechanical stress in *A. koa.*
3. To determine the level of acquired resistance in mechanically stressed *A. koa* seedlings.

The experimental results for Specific Objective 1 are described in Chapter 4. While working on isolation of RNA, an improved RNA isolation protocol was developed, and it is the subject for Chapter 3. The experimental results for Specific Objectives 2 and 3 are presented in Chapter 5 and 6, respectively.

CHAPTER 2
LITERATURE REVIEW

Inducers of disease resistance in plants: crosstalk among abiotic and biotic stress responses

2.1. Plant innate immune system

Unlike mammals, plants lack mobile specialized immune cells, such as macrophages, T cells, and B cells. However, they have various defense strategies to protect themselves from a wide range of pathogens, including insects, bacteria, and fungi. There are two modes in the plant innate immune system: microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) and effector-triggered immunity (ETI).

In MTI, cell-surface pattern-recognition receptors (PRRs) recognize various MAMPs of pathogenic and non-pathogenic microorganisms (Fig. 5). Plants can perceive invading bacteria through PRRs, such as leucine-repeat-rich receptor-kinases (LRR-RLK) called flagellin-sensing2 (FLS2) and elongation factor-Tu receptor (EFR). FLS2 recognizes flagellin proteins (flg22), and EFR recognizes elongation factor Tu (EF-Tu) of bacteria. Both receptors form heterodimers with brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1) upon the recognition of the MAMPs, such as flg22 and EF-Tu (Sun et al. 2013). Plants can also recognize fungi through other PRRs, such as chitin elicitor-binding protein (CEBiP) and chitin elicitor receptor kinase 1 (CERK1), which bind to chitin of fungal cell walls (Wan et al. 2008). Once PRRs recognize the MAMPs, they induce mitogen-activated protein kinase (MAPK) signaling cascades to express defense-related genes such as disease resistance (R) genes, pathogenesis-related (PR) genes, and genes for secondary metabolite biosynthesis. PRRs also activates cyclic nucleotide-gated channels (CNGCs), leading to the cytosolic Ca^{2+} increase. Following the elevation of cytosolic Ca^{2+} by CNGCs, calmodulin (CML) and calcium-dependent protein kinase (CDPK) activate nitric oxidase-associated protein (NOA) and respiratory burst oxidase homolog (Rboh; Kobayashi et al. 2007; Ma et al. 2008). The production of nitric oxide (NO) and reactive oxygen species (ROS) by NOA and Rboh leads to hypersensitive response (HR), causing rapid localized cell death to prevent the spread of infection (Moreau et al. 2008; Fig. 5).

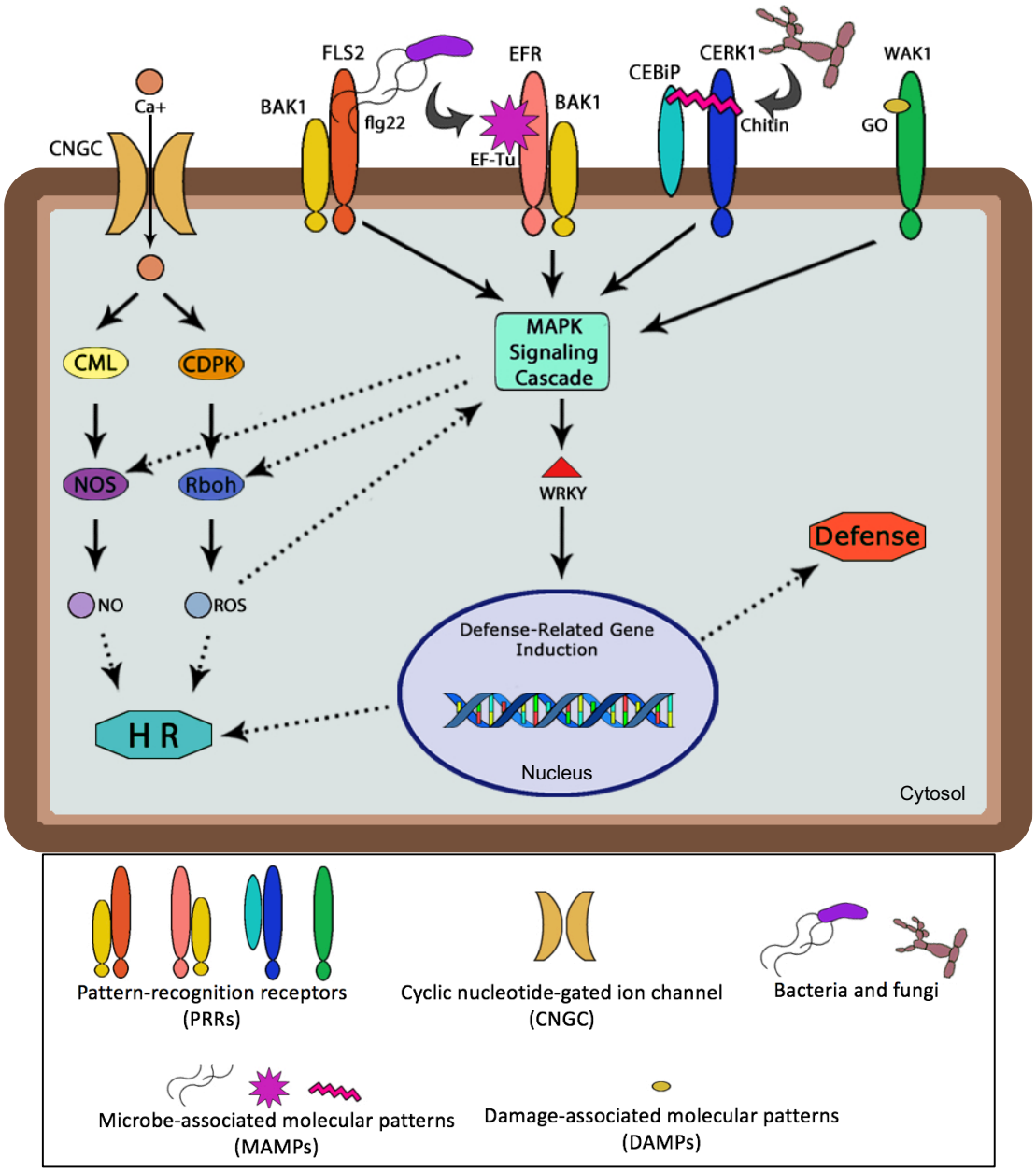

Figure 5. Simplified scheme of MAMP-triggered immunity (MTI) and DAMP-triggered immunity (DTI) in plants. Schematic of a plant cell is used to illustrate the MTI and DTI responses. MTI is initiated upon recognition of exogenous microbe-associated molecular patterns (MAMPs), such as flg22 of bacteria and chitin of fungi. DTI is initiated upon recognition of endogenous damage-associated molecular patterns (DAMPs), such as oligogalacturonic acid (OG) fragments produced as a result of cell wall degradation and ATP released outside of the cells due to cell lysis. Recognition by pattern-recognition receptors (PRRs) leads to activation of cyclic nucleotide-gated ion channels (CNGCs) and mitogen-activated protein kinase (MAPK) signaling cascade, resulting in various defense responses. Dash arrows represent multiple reaction steps. Abbreviations: flg22, flagellin 22; EF-Tu, elongation factor-Tu; FLS2, flagellin-sensing2; EFR, EF-Tu receptor; CERK1, chitin-elicitor receptor kinase 1; CEBiP, chitin-elicitor binding protein; BAK1, Brassinosteroid insensitive 1-associated receptor kinase 1; WAK1, wall-associated kinase 1; OG, oligogalacturonic acid; CML, calmodulin; CDPK, calcium-dependent protein kinase; NO, nitric oxide; ROS, reactive oxygen species; NOS, NO synthase/NO-associated protein 1; Rboh, respiratory burst oxidase homolog; HR, hypersensitive response.

There are also PRRs that can perceive endogenous damage-associated molecular patterns (DAMPs). Wounding by insect or herbivore attack or pathogen-induced cell-lysis can release DAMPS, or endogenous molecules and fragments in the extracellular space that are perceived as a 'danger' signal. For example, short oligogalacturonic acid fragments generated from cell wall degradation are a DAMP and is recognized by the wall-associated kinase (WAK1; Decreux et al. 2006; Brutus et al. 2010; Kohorn and Kohorn 2012). Extracellular ATP is another DAMP that is recognized by a lectin receptor kinase P2K1 (Tanaka et al. 2014). Recognition of DAMPs amplifies MTI signaling to establish an enhanced systemic immune response (Huffaker and Ryan 2007; Yamaguchi and Huffaker 2011; Tintor et al. 2013). Wounding facilitates the entry of pathogens into the plant tissue, which may be the reason that it initiates innate immunity system (Savatin et al. 2014). Currently, few DAMPs and their receptors have been extensively studied (Tanaka et al. 2014), and further research would be necessary to identify and understand more DAMPs.

ETI involves various resistance (R) proteins, which participate in the initiation of defense signaling networks through the recognition of effector molecules secreted by bacterial or fungal pathogens during infection (Hammond-Kosack et al. 2007; Fig. 6). Bacterial pathogens inject effectors into the cells of their hosts using type 3 or type 4 secretion systems (for review, see Escoll et al. 2016); fungal pathogens release the effectors by growing intercellularly or forming a haustorium, the feeding structure that penetrates into the cell and draws nutrients (for review, see Presti et al. 2015). In *Arabidopsis*, RMP1, RPS2, and RPS5 have been identified as R proteins that mediate the recognition of specific avirulence gene products encoded by pathogens, leading to defense signaling pathways (Boyes et al. 1988; Axtell et al. 2003; Qi et al. 2012). Also, the resistance signaling protein RAR1 was found to determine the extent of oxidative burst at infection sites, resulting in HR and pathogen containment, thereby acting as a rate-limiting regulator of early R gene-triggered defense (Muskett et al. 2002).

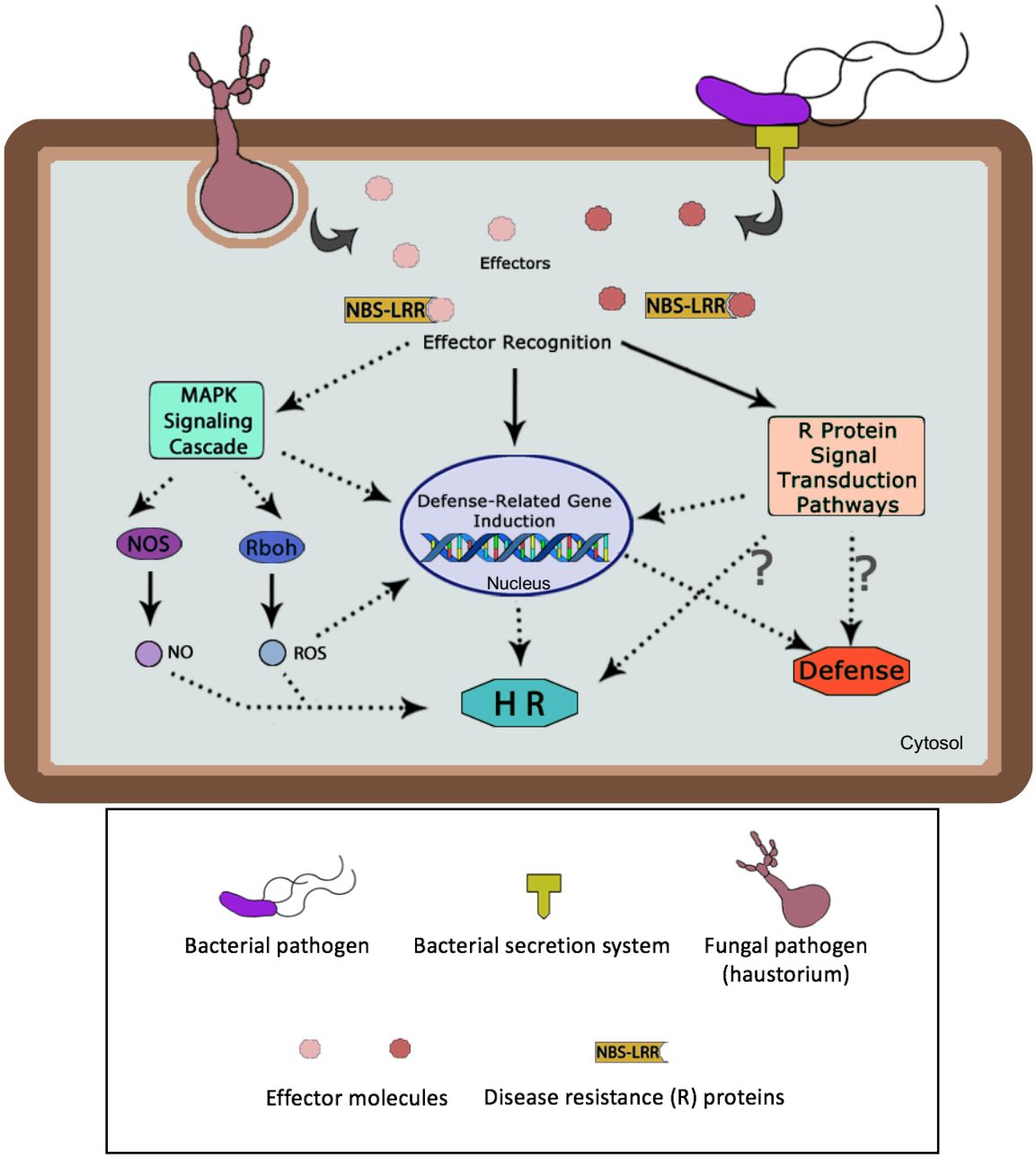

Figure 6. Simplified scheme of effector-triggered immunity (ETI) in plants. Schematic of a plant cell is used to illustrate the ETI responses. ETI is initiated when NBS-LRR, or disease resistance (R) proteins recognize effector molecules that are secreted by pathogens into the plant cells. Bacterial pathogens inject effectors into the cells of their hosts using type 3 or type 4 secretion systems; fungal pathogens release the effectors by growing intercellularly or forming a haustorium, the feeding structure that penetrates into the cell and draws nutrients. Dash arrows represent multiple reaction steps. Abbreviations: NBS-LRR, nucleotide-binding site leucine-rich repeat; NO, nitric oxide; ROS, reactive oxygen species; NOS, NO synthase/NO-associated protein 1; Rboh, respiratory burst oxidase homolog; HR, hypersensitive response.

2.2. Resistance induced by elicitors that activate MTI

Plants can develop a stronger and more effective immunity when appropriately stimulated; it is called 'induced resistance' (van der Meer et al. 2015). The two most well-known types of induced resistance are systemic acquired resistance (SAR) and induced systemic resistance (ISR). Both types appear to be induced once MTI is activated. SAR was considered to be induced when a plant is locally attacked by a pathogen, followed by HR or moderate symptom of disease (Ross 1961; Kuc and Richmond 1977); however, recent studies have shown that it is also induced when a plant is exposed to avirulent or nonpathogenic microbes besides virulent ones (for review, see Vallad and Goodman 2004). For instance, after *Musa acuminata* was inoculated with an incompatible virulent strain of *Fusarium oxysporum* that does not infect this plant, the resistance against virulent *F. oxysporum* was enhanced with higher activities of defense-related enzymes, such as phenylalanine ammonia-lyase (PAL) and peroxidase (Wu et al. 2013). SAR has been shown to last few days to the lifetime of the plant, and it is graft-transmissible (Jenns and Kuc 1979) and also epigenetically heritable (Luna and Ton 2012; Luna et al. 2012); the progeny of the plant with SAR was also shown to have enhanced resistance (Slaughter et al. 2012). The hormone salicylic acid (SA) has been shown to be associated with SAR (for review, see Conrath 2006) and at least, in *Arabidopsis thaliana* and *Nicotiana tabacum,* SA is required to activate SAR (Gaffney et al. 1993; Delaney et al. 1994).

In contrast to the pathogen-induced SAR, ISR is induced by colonization of non-infectious or symbiotic bacteria and fungi. These non-harmful microorganisms are also recognized by their MAMPs in plants. After inoculated with non-infectious microorganisms, plants develop higher resistance against infection by other pathogens. Jasmonic acid (JA) and ethylene (ET) are the plant hormones that mediate ISR. *Pseudomonas fluorescens* are frequently used in ISA studies since they are common root-colonizing bacteria that can improve plant health, or plant growth promoting rhizobacteria (PGPR; Rainey 1999). For example, when the roots of *Dianthus caryophyllus* were inoculated with a strain of *P. fluorescens,* the plants were protected from virulent *F. oxysporum* that was inoculated into the stem (Van Peer et al. 1991; Van Peer and Schippers 1992). Similar observations were made when *Cucumis sativus*, whose roots were inoculated with several PGPR strains, were infected with virulent *Colletotrichum orbiculare* (Wei et al. 1991). Like SAR, ISR may last for a long time, but no studies so far have demonstrated if ISR by beneficial microbes is heritable (for review, see Pieterse et al. 2014).

Wounding can also induce resistance called wound-induced resistance (WIR) through the production of DAMPs. As mentioned earlier, once DAMPs, such as OG and extracellular ATP, are recognized, the immune responses take place accompanied by oxidative burst and the accumulation of proteinase inhibitors and hydrolytic enzymes, such as chitinases and glucanases that protects plants to further infection (van Loon and van Strien 1999; for review, see Savatin et al. 2014). For example, in *Arabidopsis*, wounding leaves with forceps made the plant more resistant to *Botrytis cinerea* (Beneloujaephajri et al. 2013). Just like in ISR, genes involved in biosynthesis of JA and ET are rapidly induced in WIR (Reymond et al. 2000; Delessert et al. 2004); therefore, they may share the same pathways.

Because SA, JA, and ET are the main mediators of induced resistance, these phytohormones and their mimics can also enhance resistance. For example, SA mimics, β-aminobutyric acid (BABA) and benzothiadiazole (BTH) are widely used in the field as plant protectors that suppress disease symptoms caused by various pathogens (Cohen 2002; for review, see Goellner and Conrath 2008). Because of the complexity of the pathways, the plant hormones alone may not induce resistance in the same way as MAMPs, DAMPs, or beneficial microorganisms. For instance, although JA was shown to mediate WIR (Howe 2004), the application of JA alone did not activate all wound-induced genes (Reymond et al. 2000). However, unlike mammal adaptive immunity, induced resistance in plants is not pathogen-specific, so it can be effective against a broad spectrum of invaders. If infection by a fungal pathogen induces resistance, the plant typically has enhanced resistance against other fungal and bacterial pathogens besides the specific fungal pathogen. Therefore, any elicitors that can induce resistance may be useful for overall disease resistance in plants.

2.3. Resistance induced by abiotic elicitors

The recognition of MAMPs or DAMPs is not the only way for plants to enhance their disease resistance; they can also elevate their resistance through abiotic stimulation. Both abiotic and biotic stress responses are typically mediated by different plant hormones; abscisic acid (ABA) is more associated with abiotic stress responses, whereas SA, JA, and ET are more predominantly involved in biotic stress responses. However, different hormonal pathways interact with each other, resulting in either synergistic or antagonistic effects (Bostock 2005). Crosstalk between pathways for abiotic and biotic responses is complex because plants orchestrate a specific program of gene expression based on the exact environmental condition. For instance, both abiotic and biotic responses are usually accompanied with accumulation of

ROS and expression of MAPK pathway genes (Fig. 7); however, because ROS and MAPK signal transduction pathways have diverse functions, different combinations of genes and signals result in different effects. If an abiotic stress induces genes for antagonistic enzymes, such as MAPK5, it may repress resistance to pathogens since overexpression of MAPK5 was shown to decrease resistance to fungal and bacterial pathogens although it increased tolerance to drought, salt, and cold stresses and to (Xiong and Yang 2003).

Figure 7. Simplified scheme of abiotic and biotic stress responses in plants. Abiotic and biotic stresses activate various pathways that interact and inhibit one another, leading to specific responses depending on stresses. Abiotic stresses generally activate abscisic acid (ABA) signaling, while biotic stresses are more associated with ethylene/jasmonic acid (ET/JA) and salicylic acid (SA) signaling. These hormonal signaling pathways are known to inhibit each other although sometimes they have a positive interaction. Reactive oxygen species (ROS) accumulation and mitogen-activated protein kinase (MAPK) signaling cascade are induced by both biotic and abiotic stresses.

Table 1. Abiotic elicitors that induce (+) or reduce (-) disease resistance in different plants

Abiotic elicitors	Plant species	Pathogens	Resistance	Reference
Sweeping of leaf surface	*Arabidopsis thaliana*	*Botrytis cinerea*	+	Benikhlef et al. (2013)
Fan-produced wind (speed at 3 m/s)	*Phaseolus vulgaris*	*Colletotrichum lindemuthianum*	+	Cipollini (1997)
		Mites	+	
Fan-produced wind (speed at 4 m/s)	*Solanum lycopersicum*	*Fusarium oxysporum*	+	Elsharkawya et al. (2015)
Fan-produced wind (speed at < 7.3 m/s)	*Oryza sativa*	*Magnaporthe grisea*	+	Taguchi et al. (2014)
Fan-produced wind (speed at ≥ 7.3 m/s)			-	
Shaking of stems	*S. lycopersicum*	*F. oxysporum*	-	Shawish and Baker (1982)
	Linum usitatissimum	*F. oxysporum*	-	
	Pisum sativum	*F. oxysporum*	-	
Water deficiency	*Hordeum vulgare*	*Blumeria graminis*	+	Ayres and Woolacott (1980)
			-	Oerke and Schönbeck (1986)
		Cochliobolus sativus	+	
	P. vulgaris	*C. sativus*	+	
		Uromyces phaseoli	-	
		Macrophomina phaseolina	-	Mayek-Perez et al. (2002)
	Nicotiana benthamiana	*Pseudomonas syringae*	+	Ramegowda et al. (2013)
	Sorghum bicolor	*M. phaseolina*	-	Diourte et al. (1995)
	S. lycopersicum	*B. cinerea*	+	Achuo et al. (2006)
Cold stress	*H. vulgare*	*E. graminis*	-	Oerke et al. (1992)
Cold and heat stresses	*H. vulgare*	*E. graminis*	-	Oerke and Schönbeck (1986)
		C. sativus	+	
	P. vulgaris	*U. phaseoli*	-	
		C. sativus	+	
Heat stress	*H. vulgare*	*Cochliobolus sativus*	-	Sharma et al. (2007)
Acidity	*H. vulgare*	*B. graminis*	+	Wiese et al. (2004)
Salinity	*H. vulgare*	*B. graminis*	+	
	S. lycopersicum	Odium neolycopersici	+	Achuo et al. (2006)
Low light stress	*H. vulgare*	*E. graminis*	-	Oerke and Schönbeck (1986)
		C. sativus	+	
	P. vulgaris	*U. phaseoli*	-	
		C. sativus	+	

While some abiotic-stress responsive genes suppress biotic-stress responsive genes, or *vice versa*, transcriptomic studies have shown several genes related to disease resistance are expressed in response to abiotic stresses, suggesting possible overlap between the abiotic and biotic stress responses. In *Arachis hypogaea*, 25 genes among 384 tested were commonly expressed in responses to both drought stress and infection by *Aspergillus parasiticus* (Luo et al. 2005). Similarly, Shaik and Ramakrishna (2013) showed that ~30% of the genes tested in the microarray analyses of 3100 and 900 genes from *Arabidopsis* and *Oryza sativa,* respectively, were commonly expressed in both drought and bacterial stresses. Swindell (2006) identified 67 common genes in *Arabidopsis* that were up- or down-regulated in response to nine different abiotic stresses, including cold, osmotic stress, salt, drought, DNA damage, ultraviolet light, oxidative stress, wounding, and high temperature. Four of them were defense-related; although the genes for a β-1,3-glucanase and a chitinase were downregulated, the genes for a R protein and a pathogenesis-related (PR) protein were upregulated in all the nine abiotic-stress responses. On the other hand, in *Solanum lycopersicum* (*Lycopersicon esculentum*), non-wounding stress induced by a fan-produced wind upregulated genes for PR-1, acidic and basic chitinases, and PAL (Elsharkawya et al. 2015). The whole genome microarray analysis of the genes induced by bending of the stems of *Arabidopsis* showed upregulation of 14 R proteins (Lee et al. 2005). Therefore, when an appropriate gene expression cascade is induced through an abiotic stress, disease resistance can be enhanced.

How the abiotic and biotic stress signaling pathways interact may differ depending on biotic and abiotic elicitors, dosage of elicitors, timing, and perhaps on plant species (Table 1). Therefore, it is difficult to determine if abiotic elicitors would induce or suppress disease resistance. For example, Oerke and Schönbeck (1986) showed that the water deficient *Hordeum vulgare* and *Phaseolus vulgaris* were more tolerant to a biotrophic fungus and more susceptible to a necrotrophic fungus; however, it is not always the case since the water-deficient *S. lycopersicum* developed an enhanced resistance against the nectrotorophic fungus *B. cinerea* (Achuo et al. 2006). Recently, there have been more studies on non-wounding mechanical stresses as a possible elicitor of disease resistance. Gentle fan-produced wind can induce disease resistance as observed in *P. vulgaris, S. lycopersicum,* and *O. sativa* against various diseases, including *F. oxysporum* (Cipolini 1997; Teguchi et al. 2014; Elsharkawya et al. 2015); however, when the wind was too strong, it made plants more susceptible (Taguchi et al. 2014). Three plants, *S. lycopersicum, Linum usitatissimum*, and *Pisum sativum* had reduced resistance against *F. oxysporum* following gentle shaking of the stems as to imitate the motion

caused by wind (Shawish and Baker 1982); in this case, it is possible that the plants were not given the appropriate amount of the elicitor or that the response is plant-species specific. Other abiotic stresses, such as acidity, salinity, temperature, and light have been also shown to alter disease resistance (Table 1).

2.4. Mechanism of induced resistance: priming

Since exposure to a stimulus, either abiotic or biotic, can influence response to a later stimulus in plants, it indicates that plants can retain 'memory.' Plants with the innate immune memory are said to have 'trained immunity,' or to be 'primed' (van der Meer et al. 2015). Primed plants are often sensitized and can respond faster and more efficiently to stresses, thus having enhanced resistance. For example, 500 μM of SA was necessary to induce the *PAL* defense gene for the first time; however, only 10 μM was needed for the second time because the plant was sensitized (Thulke and Conrath 1998). Also, the BTH-primed *Arabidopsis* showed higher *PAL* and *PR-1* expression in response to *Pseudomonas syringae* (Kohler et al. 2002).

Although priming has been known for many years, the molecular mechanisms underlying defense priming still remains elusive. It has been proposed that priming involves biosynthesis and accumulation of enzymes that are important in signal transduction and/or amplification. Enzymes in the MAPK pathway are a potential candidate of mediators for defense priming. The mRNA transcript and protein of MAP3/6 have been identified to accumulate following SA and BTH treatments or local *P. syringae* infection in *Arabidopsis* (Beckers et al. 2009). An increased MAPK activity and defense responses were followed after the re-stimulation (Goellner and Conrath 2008). Abiotic stresses also induce the MAPK pathway, so it is possible that they also lead to accumulation of mRNA and proteins of certain MAPKs that overlap with biotic stress signaling pathways, and it would make plants ready for successive infection.

Chromosomal modifications may also take an important role in the priming mechanism because it can keep the promotor of defense-related genes 'active' for faster transcription of the genes. Alvarez-Venegas et al. (2007) has shown that the induction of WRKY70, a transcription factor activated by SA and repressed by JA (Li et al. 2004), led to methylation of the histone on the promotor of the defense gene for PR1 in *Arabidopsis*. Similarly, methylation and acetylation of histones on the promotors of WRKY29, WRKY6, and WRKY56 following BTH treatment and *P. syringae* infection were observed in *Arabidopsis* (Jaskiewicz et al. 2011). These modifications did not activate transcription of the WRKYs until the plants were infected again. Histone

modifications have been also observed following environmental stresses. For instance, histones on the regions of drought-inducible genes were modified in response to drought stress in *Arabidopsis* (Kim et al. 2008; Kim et al. 2012). Chromatin modification is likely to be one of the mechanisms to store the 'memory' of both biotic and abiotic responses so the defense pathways are induced faster for the future challenges.

This epigenetic modification is also shown to be inherited from the primed *Arabidopsis* parents to the next stress-free generation, which also showed induced resistance to various pathogens (Luna et al. 2012; Luna and Ton 2012). In the second-generation offspring, the histones on the promotors of SA-induced genes *PR1*, *WRKY6*, and *WRKY53* were acetylated, and the histone on the promotor of JA-induced gene, plant-defensin1.2 (*pdf1.2*), were methylated (Luna et al. 2012). Histone methylation and acetylation may be a way to transmit immunity to the next generation in plants.

CHAPTER 3
METHODS

Development of an improved RNA extraction from *Acacia koa*

3.1. *Introduction*

Acacia koa is a leguminous timber wood tree endemic to the Hawaiian Islands. With its beautiful texture, hardiness, and carving quality of the wood, it is the most economically important tree in Hawai'i (Yanagida et al. 2004; Baker et al. 2009). Similarly, *Leucaena leucocephala* is a tree legume, which is widely used for agroforestry (Brewbaker et al. 1990). For genetic studies of *A. koa* and *L. leucocephala* using molecular biology techniques such as complementary DNA (cDNA) library construction, reverse transcription-polymerase chain reactions (RT-PCR), and microarray analysis, it is a critical step to extract high-quality RNA in sufficient quantity. The isolation of RNA from these woody species poses a particular challenge in that they often contain high levels of polysaccharides and phenolic compounds that can co-purify with RNA and inhibit enzymatic manipulations (Loomis 1974; Wilkins and Smart 1996; Nassuth et al. 2000). Many existing protocols for plant RNA extraction, like TRIzol, cetyltrimethyl-ammonium bromide (CTAB), and Qiagen RNeasy® kits, cannot remove these compounds completely, which lowers the quality and quantity of extracted RNA and affects the downstream results of molecular biology techniques. As a result, a variety of methods have been developed based on the modification of traditional extraction methods, such as modified CTAB and TRIzol protocols developed by Gambino et al. (2008) and Jordon-Thaden et al. (2015) to improve RNA extraction on polyphenol-rich grapevines and woody plants.

It is often difficult to obtain sufficient amounts of high-quality RNA from seedlings of *A. koa*, especially from hardier stems. This chapter reports a modified RNA extraction method that utilizes a combination of the Qiagen RNeasy® kit and Takara Fruit-mate™. The results of this modified extraction method yielded sufficient quantity of high-quality RNA from seedlings of *A. koa,* and also another woody legume, *Leucaena leucocephala*.

3.2. Materials and Methods

3.2.1. Plant material

Seeds of *A. koa* were collected from the Maunawili sub-center of Hawai'i Agriculture Research Center (HARC), Kailua, Hawai'i. Seeds of *L. leucocephala* were collected from the Waimanalo Research Station of University of Hawai'i, Waimanalo, Hawai'i. Seeds of both

species were immersed in sulfuric acid for 10 min for scarification, rinsed with water, and incubated in petri dishes with wet filter paper at 28ºC until they germinated (3-5 days; Rushanaedy et al. 2012). The resulting germinated seedlings were then planted into a tray containing a vermiculite-perlite mixture and maintained at 25°C ± 2°C with a 16/8-h light/dark photoperiod with an irradiance of 30 μmol s^{-1} m^{-1} for six months. Leaves were removed, and stems were collected and immediately placed in liquid nitrogen prior to RNA extraction.

3.2.2. RNA extraction

Stem tissues of *A. koa* and *L. leucocephala* were ground to fine powder in liquid nitrogen with a motor and a pestle. Mortars and pestles used to isolate RNA were baked for six hours at 300 °C before use. Plasticware was autoclaved prior to use, and solutions used to isolate RNA were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) and autoclaved before use. Five RNA extraction methods were compared. Fifty milligrams of the ground tissue from each sample of *A. koa* was used for RNA extraction. The extracted RNA was eluted or dissolved in 30 μL RNase-free water in each method. Three replicates were made for each method. The five methods used were as follows:

(1) The modified extraction method: 100 μL of Fruit-mate™ (cat. no. 9192, Takara, Japan), 400 μL of Buffer RLT from the RNeasy® Plant Kit (cat. no. 74904, Qiagen, Germany), and 5 μL of β-mercaptoethanol were mixed. Mixing Fruit-mate™ with Buffer RLT formed white cloudy precipitates; it was mixed well, and 500 μL of the solution with the precipitates was added to the 50 mg of the powdered stem tissue in a 1.5 mL Eppendorf tube. The sample was vortexed vigorously. The remaining steps of RNA preparation were carried out according to Qiagen's instructions for the RNA purification from plant cells (https://www.qiagen.com/), except the additional washing step with 75% ethanol after the second wash with Buffer RPE from the RNeasy® Plant Kit.

(2) The RNeasy® extraction: the RNeasy® Plant Mini Kit (cat. no. 74904, Qiagen, Germany) was carried out as described in the manufacturer's instructions.

(3) The Fruit-mate™ extraction (cat. no. 9192, Takara, Japan): Protocol 1 of the manufacturer's instructions was followed with the exception of the substitution of 'RNAiso Plus' (Takara) with TRI Reagent BD (cat. no. T3809, Sigma-Aldrich, MO, USA).

(4) The TRIzol method: TRI Reagent BD was used according to the manufacturer's instructions.

(5) The CTAB method: it was carried out as described by Chang et al. (1993).

3.2.3. Quantitative and qualitative analysis of RNA

The quantity and quality of the RNA were assessed at wavelengths of 230, 260, and 280 nm using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, DE, USA). To confirm the quality, the RNA was analyzed based on 'gel-like' images and RNA Integrity Numbers (RIN) obtained through an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Two-sample t-test was performed to determine if the quantity and quality of RNA obtained with the modified protocol were significantly different from the ones obtained with the other four methods.

3.2.4. Downstream application: reverse transcription PCR (RT-PCR)

The extracted RNA was treated with TURBO DNAfree Kit (Ambion, CA, USA) to remove any genomic DNA contamination, and cDNA was synthesized from 500 ng of RNA using M-MLV Reverse Transcriptase (Promega, WI, USA) with random hexamers. PCR amplification was performed using primers for a conserved region of actin (forward: 5′-TGCTGGACGTGACCTTACTG; reverse: 5'-GAACCACCGATCCAGACACT), which generate a fragment of 492 bp. Each sample was analyzed in a 20 µL PCR reaction consisting of 0.25 µL forward primer (10 µM), 0.25 µL reverse primer (10 µM), 10 µL GoTaq® Colorless Master Mix (Promega), and 1 µL of the synthesized cDNA from the first step of the reaction. Reaction conditions were 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, with a final annealing step at 72 °C for 5 min. PCR products were analyzed by electrophoresis on 1% agarose gels (40 mM Tris-acetate, 1 mM EDTA, 0.5X GelRed [Biotium, CA, USA]) and visualized using UV light. Similarly, no-reverse transcription (no-RT) control was performed to monitor genomic DNA contamination using the extracted RNA instead of the synthesized cDNA as a template.

3.3. *Results and Discussion*

3.3.1. Quality and quantity of RNA

Compared to the other four methods, the modified method yielded sufficient amounts of high-quality RNA (Table 2). Typically, RNA with the A_{260}/A_{280} and A_{260}/A_{230} ratio $\geq$2.0 is considered pure and required for accurate and consistent results in downstream applications (Bilgin et al. 2009; Johnson et al. 2012). The study showed that the modified method was optimal for producing reproducible good results. Among the five protocols tested in this report,

the modified method yielded the best-quality RNA with the A_{260}/A_{280} and A_{260}/A_{230} ratios greater than 2.

The total RNA extracted with the modified protocol also had an RNA Integrity Number (RIN) of 8.0 for *A. koa* and 6.8 for *L. leucocephala* with clear, distinct bands of 28S and 18S rRNA without degradation, which are the indicative of intact RNA (Fig. 8). According to Fleige and Pfaffl (2006), obtaining RNA with a high RIN is important as it has been shown to affect the results of quantitative RT-PCR. An RIN greater than 6.5 is generally considered to be acceptable as a quality index for molecular studies (Durrenberger et al. 2010). The RIN increased significantly in the modified protocol compared to the other protocols (Table 2). The 28S/18S ratio is also a common criterion to evaluate RNA integrity; however, no significant correlation was observed between the 28S/18S ratio and the quantitative RT-PCR performance (Fleige and Pfaffl 2006), and according to Copois et al. (2007), the 28S/18S ratio even can be a misleading indicator of RNA integrity. Therefore, this ratio was not used to assess the RNA integrity. The modified method also yielded over 200 µg of RNA from one gram of stem tissue of both *A. koa* and *L. leucocephala*; it is higher than or within the range of the quantity obtained from previously published modified RNA extraction protocols for woody plant species (Chang et al. 1993; Gambino et al. 2008). The combination of the two reagents creates a synergistic effect in RNA extraction, but mechanisms are not known; the compositions of both reagents are also not known as they are proprietary reagents.

Although the RNA yield from *L. leucocephala* using the modified method was 25% lower than using the Fruit-mate™ method, the latter method had a RIN of 3.0 and produced a smear in the gel-like image, which indicated partial degradation. The TRIzol method produced low-quality RNA from both legume species (Table 2). These RNA samples did not produce any visible bands of 18S and 28S rRNA in the gel-like image (Fig. 8), indicating that they were degraded. Similarly, although the CTAB method produced a yield over 800 µg RNA/g plant tissue, the RNA was partially degraded; the gel-like picture showed smearing and the 28S rRNA band was fainter. Its RIN also indicated partial degradation of RNA, and such partially degraded RNA is not suitable for applications like cDNA library construction and quantitative PCR analysis (Fleige and Pfaffl 2006).

Table 2. Quantity and quality of total RNA extracted by five methods from woody stems of *Acacia koa* and *Leucaena leucocephala* (mean ± SE, n = 3)

Extraction Method	Quantity (µg/g plant)	A_{260}/A_{280} Ratio	A_{260}/A_{230} Ratio	RIN
A. koa				
Modified	213.2 ± 33.9	2.14 ± 0.01	2.09 ± 0.15	8.0 ± 0.0
RNeasy®	102.1 ± 5.0*	1.98 ± 0.01**	0.94 ± 0.02**	6.8 ± 0.1*
Fruit-mate™	40.4 ± 3.5*	1.79 ± 0.01**	0.28 ± 0.03**	3.0 ± 0.6**
TRIzol	81.8 ± 14.8*	1.58 ± 0.07**	0.32 ± 0.02**	2.6 ± 0.0**
CTAB	16.6 ± 3.8*	1.83 ± 0.03*	1.85 ± 0.20	ND
L. leucocephala				
Modified	206.7 ± 24.5	2.14 ± 0.00	2.11 ± 0.02	6.8 ± 0.1
RNeasy®	19.4 ± 6.04**	2.09 ± 0.05	0.70 ± 0.06**	ND*
Fruit-mate™	275.3 ± 16.7*	0.28 ± 0.01**	0.97 ± 0.06**	5.2 ± 0.1**
TRIzol	129.1 ± 18.6*	1.58 ± 0.03**	0.32 ± 0.06**	ND
CTAB	831.2 ± 47.2**	1.85 ± 0.01*	2.29 ± 0.02**	4.5 ± 0.9*

'ND' indicates that RIN value was not detectable due to the RNA concentrations being too low. An asterisk (*) indicates significant difference ($p < 0.05$) with the modified protocol. Two asterisks (**) indicate significant difference ($p < 0.01$) with the modified protocol.

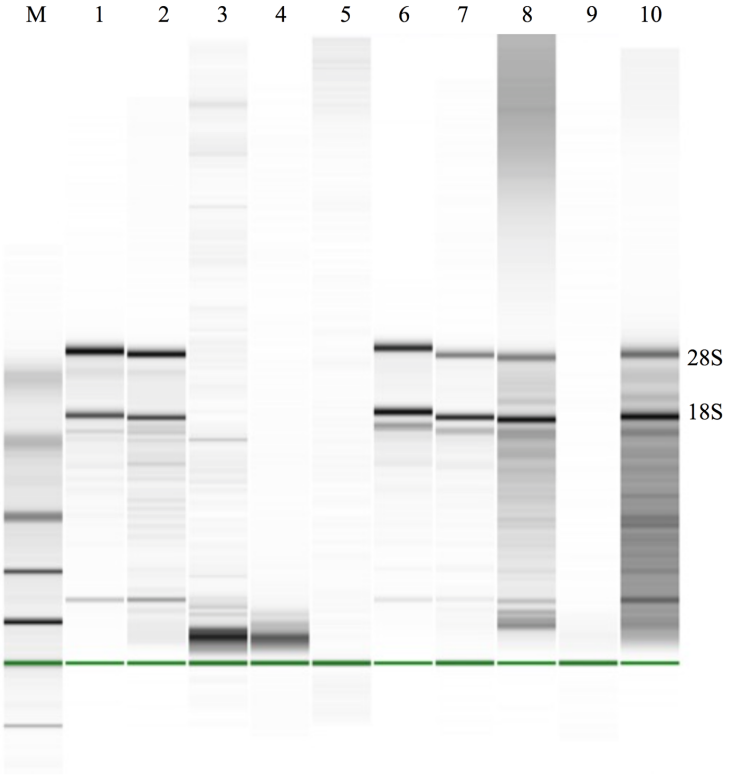

Figure 8. Gel-like image of total RNA obtained from Agilent Bioanalyzer. Total RNA of *Acacia koa* was extracted by (lane 1) the modified method, (lane 2) the Qiagen RNeasy® method, (lane 3) the Fruit-mate™ method, (lane 4) the TRIzol method, and (lane 5) the CTAB method. Total RNA of *Leucaena leucocephala* was extracted by (lane 6) the modified method, (lane 7) the Qiagen RNeasy® method, (lane 8) the Fruit-mate™ method, (lane 9) the TRIzol method, (lane 10) the CTAB method. M - a marker. Clear, distinct bands of 28S and 18S rRNA are the indicative of intact RNA.

3.2.2. Reverse transcription PCR (RT-PCR) analysis of RNA

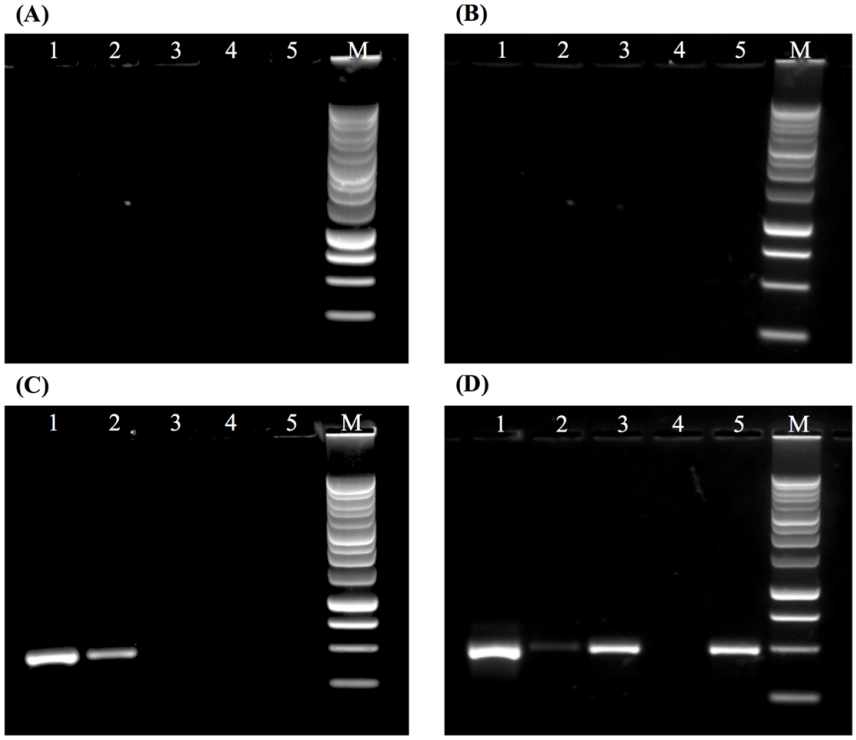

Figure 9. Reverse transcription PCR (RT-PCR) analysis of RNA extracted from *Acacia koa* and *Leucaena leucocephala.* An actin gene fragment was amplified for this analysis. No-RT-PCR controls were performed with RNA extracted from (A) *A. koa* and (B) *L. leucocephala* as template, and RT-PCR was performed with cDNA synthesized from RNA of (C) *A. koa* and (D) *L. leucocephala*. RNA used was extracted by (lane 1) the modified method, (lane 2) the Qiagen RNeasy® method, (lane 3) the Fruit-mate™ method, (lane 4) the TRIzol method, and (lane 5) the CTAB method. M - a 1 kb DNA marker. All the RNA samples were found to be free from DNA contamination since no-RT controls did not show any amplification for both *A. koa* and *L. leucocephala* (A and B). The RNA extracted with the modified protocol had the best amplification of the actin fragment in both plant species (C and D).

The RT-PCR results showed that the modified method produced high-quality RNA usable for downstream applications. All the RNA samples were found to be free from DNA contamination because no-RT controls in the RT-PCR assay did not show any amplification for both *A. koa* and *L. leucocephala* (Fig. 9A and 9B). From *A. koa* samples, RT-PCR with the RNA extracted with the modified protocol successfully amplified the actin fragment (Fig. 9C). Although RT-PCR of the *A. koa* RNA obtained from the RNeasy® method also produced the actin fragment, the modified protocol resulted in greater amplification compared to the RNeasy® method. This result is consistent with the quality and integrity of RNA, as the RNeasy®-extracted RNA had a low A_{260}/A_{230} ratio, indicating presence of impurities that may interfere with PCR amplification. Moreover, the *A. koa* RNA samples extracted with the other three methods were highly degraded and produced no amplification (Fig. 9C). Although RT-PCR with the *L.*

leucocephala RNA samples extracted with all the protocols except the TRIzol method amplified the actin fragment, the amplification was much more with the modified method (Fig. 9D). This result was similar to that observed for *A. koa.*

3.4. *Conclusion*

The results demonstrated that the modified extraction method combining RNeasy® and Fruit-mate™ consistently yields a sufficient quantity of high-quality RNA from the two woody legume species *A. koa* and *L. leucocephala* while the other methods failed to produce such high-quality RNA. RT-PCR results also indicated that the RNA extracted from this method is usable for downstream applications. This improved protocol is rapid and easy, and it will facilitate genetic studies of *A. koa, L. leucocephala*, and other leguminous tree species.

CHAPTER 4
RESULTS FOR SPECIFIC OBJECTIVE 1

Illumina-based *de novo* transcriptome analysis and identifications of genes involved in plant immunity and phenylpropanoid biosynthesis in *Acacia koa*

4.1. *Introduction*

Acacia koa is an important leguminous tree endemic to the Hawaiian Islands. The native *A. koa* forests are broadly distributed across all five major Hawaiian Islands (Wagner et al. 1999). The *A. koa* populations in these islands are genetically diverse and can be divided into morphologically distinguishable groups of koa, koaia, and an intermediate type (Adamski et al. 2012). *Acacia koa* serves as an ecologically and economically vital resource for the Hawaiian Islands. It provides habitat for many native fauna and flora (Sakai 1988; Whitesell 1990; Elevitch et al. 2006). In addition, due to the beautiful texture, hardiness and carving quality of the wood, the *A. koa* timber, referred to as koa or Hawaiian mahogany, is a high priced commodity with a current market value of up to $125 per board ft (Baker et al. 2009). *Acacia koa* wood is used for fine furniture, decorative items, musical instruments, and jewelry. The gross value of the *A. koa* timber and wood products produced is estimated to be in the range of $20-$30 million annually (Yanagida et al. 2004; Baker et al. 2009).

Several surveys since 1954 have shown that a wilt- and dieback-inducing fungal pathogen, later identified as *Fusarium oxysporum* f. sp. *koae,* has been the major cause of decline of *A. koa* in the native Hawaiian forests (for review, see Gardner 1996). In recent years, there has been a growing interest in re-establishment of *A. koa* in Hawai'i as a commercial plantation species on abandoned sugarcane and pineapple lands, but such attempts are hampered due to the destructive wilt disease (Newell and Buck 1996; Pejchar and Press 2006). To save *A. koa* forestry from the infection, selection for resistance is necessary. One method of selection for resistance is selecting seeds for planting from resistant mother trees. However, because of the highly cross-pollinating nature of *A. koa,* all progenies of the resistant trees are not resistant. Moreover, under natural condition, it is difficult to distinguish disease resistant trees from disease escape trees. Therefore, simply selecting mother trees for the source of seeds alone cannot ensure identification of truly resistant *A. koa*. To select for resistant genotypes, identification of genes for resistance will be essential. Resistance gene sequences can be used as markers for selection of resistant seedlings. Once genetic markers are developed, marker-assisted selection will significantly accelerate the *A. koa* improvement program.

Rushanaedy et al. (2012) observed that expression of chitinase genes following inoculation by pathogenic strains of *F. oxysporum* can be used as markers for resistance in *A. koa.* To identify large numbers of genes for resistance in *A. koa*, sequencing of the genome will be necessary. However, given that *A. koa* is proposed to be an allotetraploid (Shi 2003), it is substantially challenging to sequence and assemble its complex genome (for review, see Hamilton et al. 2012). For such cases, transcriptome sequencing provides an alternative to the whole genome sequencing. The newly developed high-throughput next generation sequencing (NGS) technology, such as Roche/454 Genome Sequencer FLX instrument, the ABI SOLiD System, and the Illumina Genome Analyzer, is a rapid and cost-efficient tool for *de novo* transcriptome sequencing for non-model organisms like *A. koa*.

In the present study, Illumina sequencing technology was utilized to characterize the transcriptome of *A. koa*. The objectives of this study were to enrich the gene resource of *A. koa* with the sequencing data, to identify the transcripts involved in disease resistance in *A. koa*, and to develop putative simple sequence repeats (SSRs) molecular markers that could also be used for selection of superior *A. koa* seedlings. To the best of the knowledge, this study is the first exploration to characterize the transcriptome of *A. koa* through Illumina sequencing technology. The transcriptome sequencing of *A. koa* will offer valuable sequence resource and contribute to further research on functional genomics and improvement of *A. koa*.

4.2. *Materials and Methods*

4.2.1. Plant materials and RNA extraction

Two-and-a-half-month old A. koa seedlings were obtained from the Maunawili sub-center of HARC, Kailua, HI via flash-freezing in liquid nitrogen on site. Total RNA was extracted from the whole seedlings using Qiagen RNeasy® Plant Kit (Valencia, CA, USA) and Fruit-mate™ (Takara, Japan) as described by Ishihara et al. (2016). The extracted RNA was purified with TURBO DNA-free Kit (Ambion). The quality and quantity of the RNA were assessed using NanoDrop Spectrophotometer (ND-1000).

4.2.2. Library construction, sequencing, and assembly

Cofactor Genomics, St. Louis, MO conducted cDNA library construction, sequencing and assembly. Sequencing was performed through the Illumina platform (Illumina Genome Analyzer IIx) with 60 bp paired-end reads. The quality of the raw reads was assessed through FASTQC to make sure more than 90% of the bases have Q20 or higher and were assembled using a *de*

novo assembly program Trinity (http://trinityrnaseq.sourceforge.net/) (Grabherr et al. 2011). The resulting assembled sequences were defined as unigenes. Assembled sequences with lengths ≥ 200 bp were included in the downstream analysis.

4.2.3. Functional annotations of unigenes

The assembled unigene sequences were compared against multiple protein databases, including the NCBI non-redundant (nr) database, the Swiss-Prot database, the Translated European Molecular Biology Laboratory (TrEMBL) database, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the Clusters of Orthologous Group (COG) database, through the Basic Local Alignment Search Tool (BLAST) algorithm with a cutoff E-value of 1E-3, using the doblast server of the Noble Foundation (http://bioinfo3.noble.org/doblast/) and the WebMGA server (http://weizhonglab.ucsd.edu/metagenomic-analysis/) (Wu et al. 2011). Gene names were assigned to each query based on the highest sequence similarity. A Java program Blast2Go (Conesa et al. 2005) was utilized to assign Gene Ontology (GO) functional categories for the annotated unigenes. The COG database, which classifies orthologous gene products, was used to categorize the annotated unigenes into 26 general functional groups. With the KEGG database, which contains systematic analysis of biochemical pathways and functions of the gene products, unigenes involved in the monolignol biosynthesis pathway were identified. The BLASTX analysis was performed to confirm the sequence identities of some unigenes in the ortholog groups and to detect unigenes with a complete Open Reading Frame (ORF). NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/) was used to determine the ORFs and the protein sequences of the unigenes.

4.2.4. Identification of simple sequence repeats (SSRs)

For development of new molecular markers, the annotated unigenes were used to identify potential simple sequence repeats (SSRs). With the MISA Perl script (http://pgrc.ipk-gatersleben.de/misa/), motifs of di- to hexanucleotide with a minimum of four repetitions and compound motifs, in this case, motifs interrupted by sequences of up to 100 bp, were also identified.

4.3. *Results*

4.3.1. Sequence analysis and assembly

There are only a limited number of nucleotide sequences publicly available for *A. koa*. To enrich the gene resource of *A. koa* with the sequencing data and to identify the transcripts that may be related to disease resistance in *A. koa*, transcriptome sequencing was performed. In this study, a total of 88,983,363 paired-end raw reads were generated from a 250-bp insert library. These reads contained 97.66% Q20 bases (base quality 20) and were used for *de novo* assembly. The raw reads were deposited on the NCBI Sequence Read Archive (SRA) with an accession number SRR1686818. Using the Trinity *de novo* assembly software, 85,533 unigenes were generated with a total length of 45.82 Mb, an average length of 640.97 bp, and an N50 length of 1,068 bp (Table 3). Of these, 15,022 (17.56%) were >1 kb, 14,090 (16.47%) were 500-999 bp, and 56,421 (65.96%) were 200-499 bp.

Table 3. Summarized assembly statistics for unigenes in *Acacia koa*

Stats	Number
Total number of paired-end reads	88,983,363
Total number of assembled unigenes	85,533
Total length of unigenes (bp)	54,824,004
Mean length of unigenes (bp)	641
Median length of unigenes (bp)	345
Max length of unigenes (bp)	13,405
N50 length of unigenes (bp)	1,068

4.3.2. Functional Annotation

The 85,533 unigenes were searched against diverse protein databases, including the nr database, the Swiss-Prot database, the TrEMBL database, the KEGG database, and the COG database, using the BLAST algorithm (E-value < 1E-3). The annotation with the TrEMBL database had the highest aligned unigenes (46,146 unigenes), followed by the annotation with the nr database (45,800 unigenes). With the two databases combined, a total of 46,782 unigenes (54.69%) were annotated. The number of unigenes that showed homology with sequences in the Swiss-Prot, KEGG, and COG databases were 33,113, 26,024, and 20,288, respectively. Overall, a total of 47,038 unigenes (54.99%) were successfully annotated using nr, TrEMBL, Swiss-Prot, KEGG, and COG (Table 4). Among the unigenes annotated in the nr and TrEMBL databases, 50.24% and 42.75%, respectively, had an E-value < 1.0E-50, showing strong homology; however, only 33.53% of the unigenes annotated in the Swiss-Prot database had an E-value < 1.0E-50 (Fig. 10).

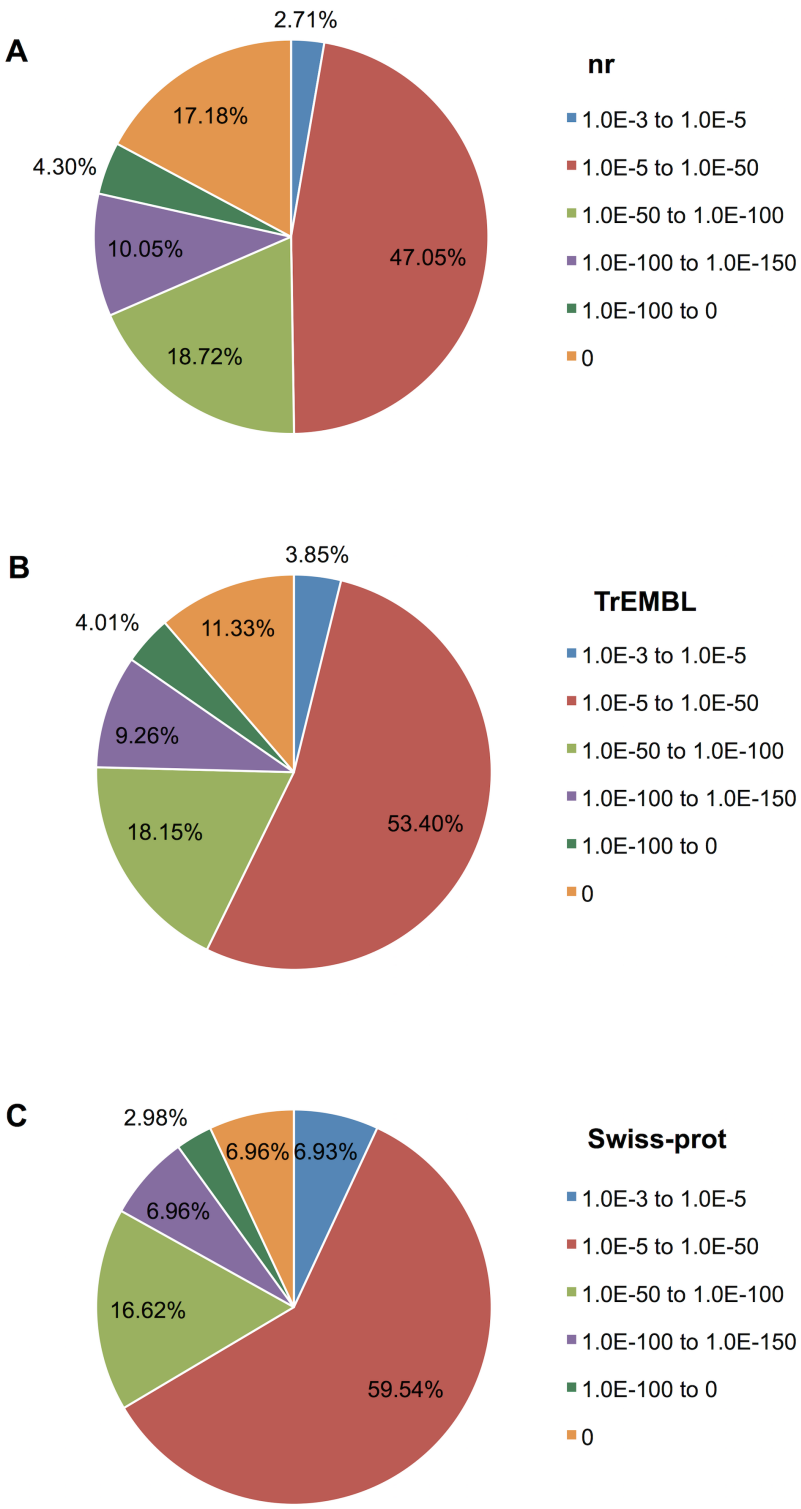

Figure 10. E-value distributions of annotated *Acacia koa* unigenes. The E-values of the highest-scored BLAST hit was identified for each unigene by aligning against (A) the nr protein database, (B) the TrEMBL protein database, and (C) the Swiss-Prot protein database for each unigene.

Table 4. Summary for the annotation of unigenes of *Acacia koa* (cutoff < 1.0E-3)

	Number of unigenes	Number of functional categories
Gene annotation against nr	45,800	-
Gene annotation against Swiss-Prot	33,113	-
Gene annotation against TrEMBL	46,146	-
Gene annotation against KEGG	26,024	-
Total gene annotation against nr and TrEMBL	46,782	-
GO annotation for nr and TrEMBL protein hits	20,884	52
KEGG pathway mapping for nr and TrEMBL protein hits	12,646	208
COG functional classification for nr and TrEMBL protein hits	18,320	26
Total annotated unigenes	46,891	-

The annotation rate decreased as the lengths of unigenes decreased; 98.16% of unigenes with the length of ≥ 1,000 bp showed homologous matches, whereas the annotation rates for unigenes with a length of 500-999 bp and unigenes < 500 bp were 78.78% and 37.60%, respectively (Fig. 11). The majority of the unigenes without annotations from the nr and TrEMBL databases were < 500 bp, as 62.25% and 28.59% were 200-299 bp and 300-499 bp, respectively, totaling 90.84%. The reason for this was most likely their short sequence lengths, resulting statistically insignificant matches.

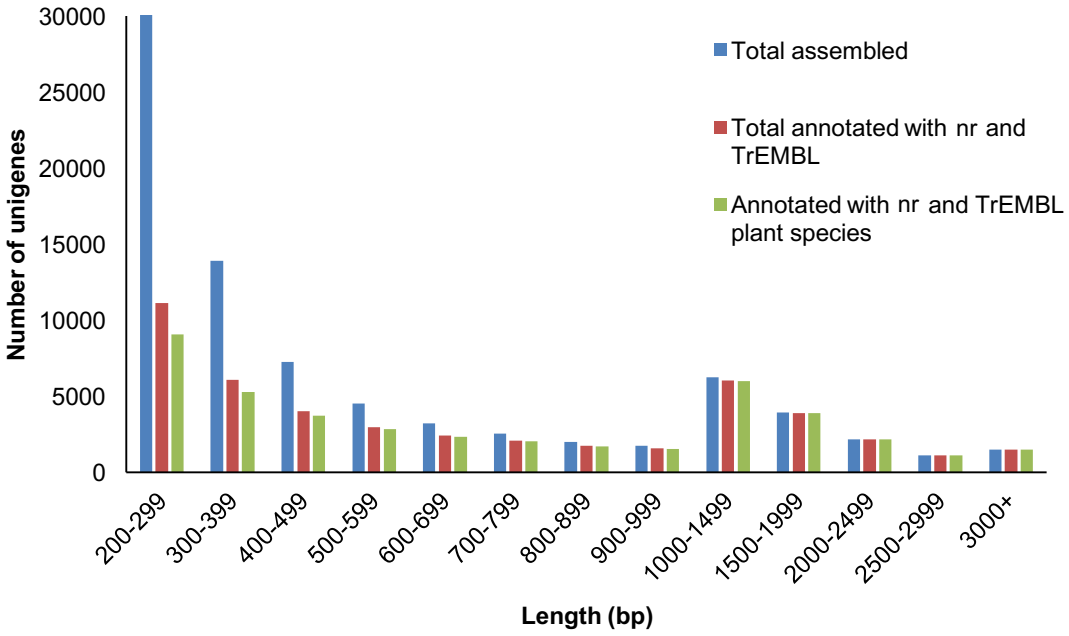

Figure 11. Length distributions of assembled unigenes of *Acacia koa*. Blue bars represent the total number of assembled unigenes. Red bars represent the total number of unigenes annotated by nr and TrEMBL. Green bars represent the total number of unigenes that have high similarities to known plant proteins. The two peak-pattern of the graph is due to two ranges of data used; the distribution range was 100 bp for unigenes with lengths of up to 1 kb, and 500 bp for unigenes above 1kb.

For the plant species distribution, the most represented species in the unigenes aligned in the nr database were all legumes: *Glycine max* (32.25%), *Cicer arietinum* (13.52%), and *Medicago truncatula* (8.02%; Fig. 12A). Similarly, more than half of the unigenes that matched with sequence in the TrEMBL database showed homology with legumes, including *G. max* (47.21%) and *M. truncatula* (8.72%; Fig. 12B). Since the Swiss-Prot database contains only manually reviewed protein sequences, a higher percentage of the unigenes (76.62%) showed homology with the well-studied *Arabidopsis thaliana* sequences (Fig. 12C). Considering the E-value and plant species distributions, the annotations of the unigenes with the nr and TrEMBL databases gave consistent results. The 46,782 unigenes annotated from the nr and TrEMBL databases were used for further analysis. Additionally, a total of 3,473 unigenes (3,262 and 3,442 annotated with the nr and TrEMBL databases, respectively) had homology to sequences with non-plant origins, such as *Staphylococcus* and *Drosophila*. Approximately 90.09% (3,128 unigenes) of those were 200-499 bp in length; 9.04% (314 unigenes) were 500-999 bp, and 0.86% (30 unigenes) were ≥ 1,000 bp (Fig. 11). These sequences were considered contaminants and removed, and the remaining 43,309 unigenes were used for sequence classifications.

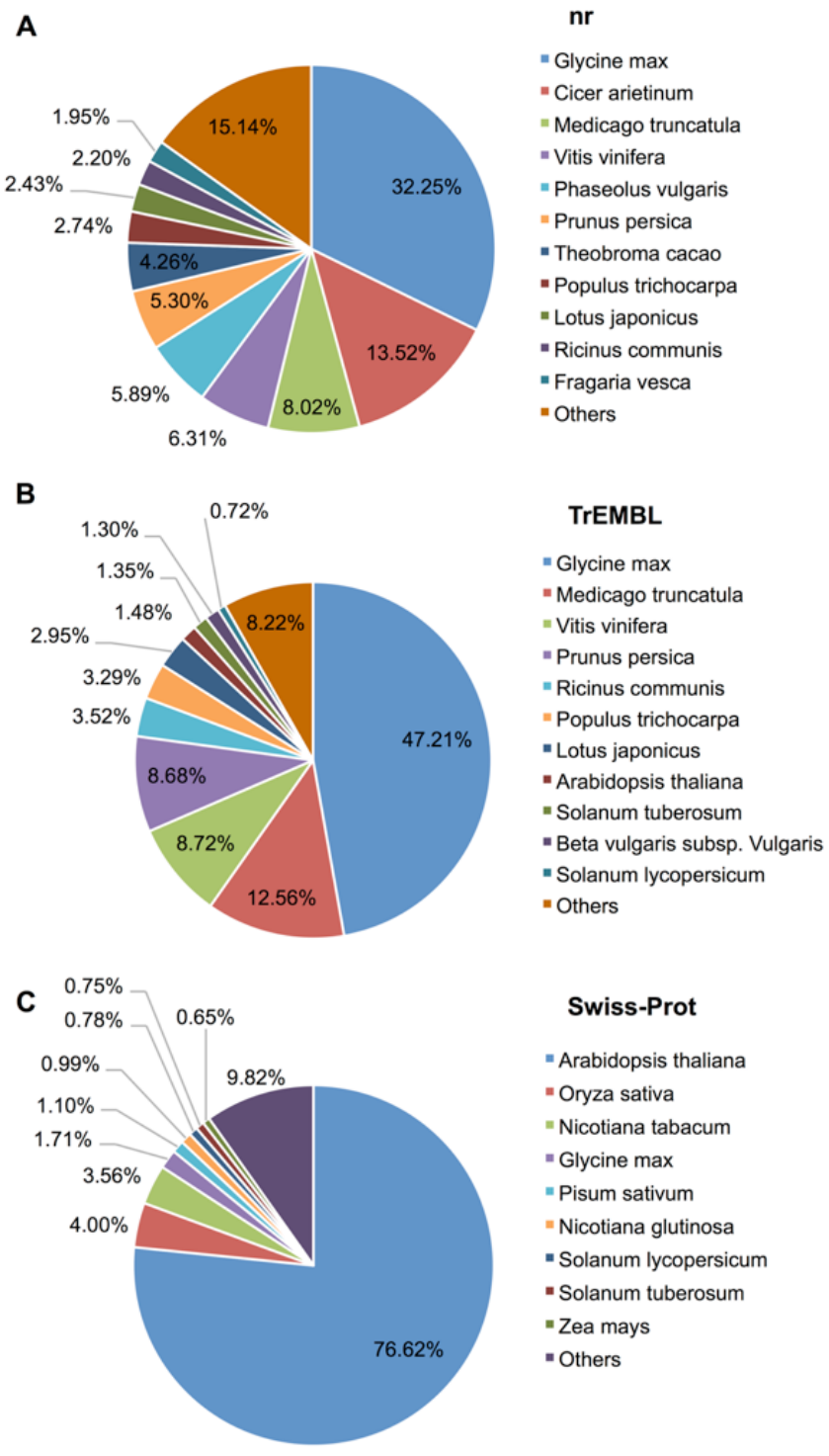

Figure 12. Top hit plant species distribution of unigenes of *Acacia koa*. The unigenes were annotated against (A) the nr protein database, (B) the TrEMBL protein database, and (C) the Swiss-Prot protein database.

4.3.3. Gene Ontology (GO) classification

Of the 43,309 unigenes, 20,884 were classified into 3 GO functional categories: biological process, cellular component, and molecular function (Fig. 13). In the biological process category, the unigenes were further clustered into 20 subcategories. Of those, the largest was metabolic process (23.99%); the second was cellular process (19.62%), and the third was single-organism process (14.25%). Under the cellular component category, the unigenes were assigned to 16 subcategories; the most abundant classes were cell (21.85%), cell part (21.84%), and organelle (15.150%). The unigenes under the molecular function category were sorted into 6 subcategories; the most represented ones were binding activity (44.33%), catalytic activity (42.69%), and transporter activity (4.76%).

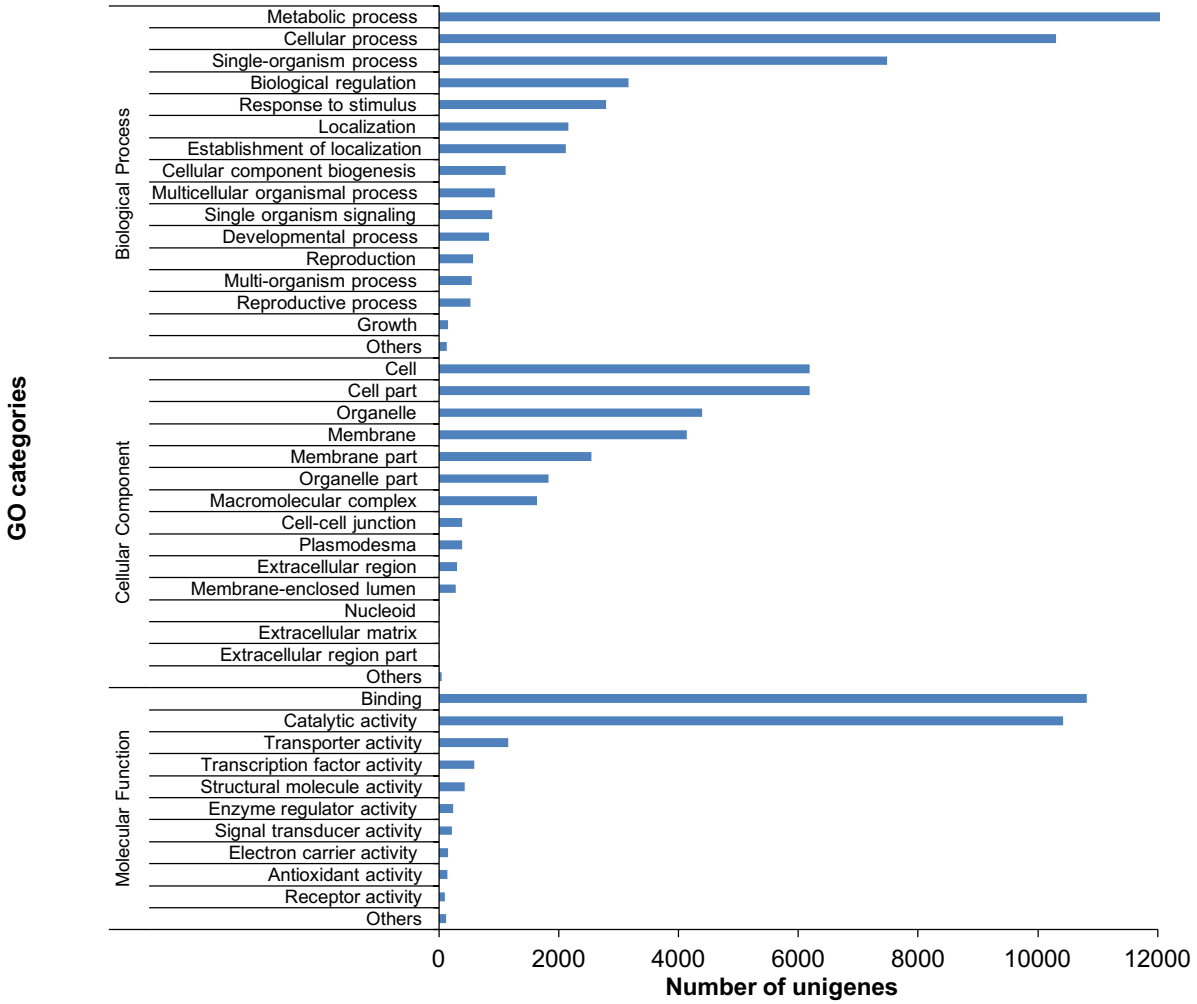

Figure 13. Gene Ontology (GO) functional categorization of the unigenes of *Acacia koa* annotated against the nr and TrEMBL databases. A total of 20,884 unigenes were classified into 3 main GO categories and 42 sub-categories.

4.3.4. Clusters of Orthologous Groups (COG) classification

Using the COG database, 18,320 unigenes of the 43,309 annotated ones were classified into 26 functional categories (Table 4; Fig. 14). Some of the unigenes were classified into more than one category. The category for 'signal transduction mechanisms' (3,224 unigenes) represented the largest group, followed by 'general function prediction only' (2,358 unigenes), 'posttranslational modification, protein turnover, chaperones' (1,979 unigenes), 'transcription' (1,242 unigenes), 'function unknown' (1,218 unigenes), 'carbohydrate transport and metabolism' (1,116 unigenes), 'intracellular trafficking, secretion, vesicular transport' (1,093 unigenes), and 'secondary metabolites biosynthesis, transport, and catabolism' (980 unigenes).

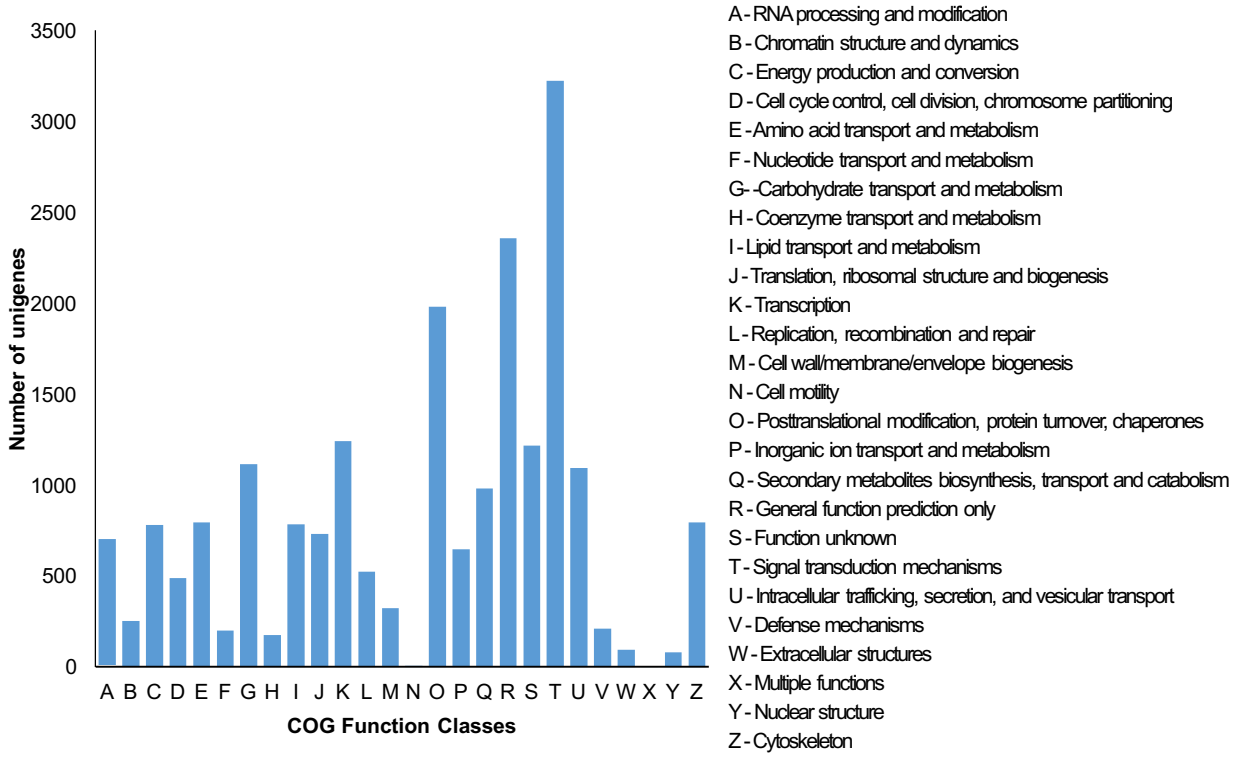

Figure 14. Clusters of Orthologous Groups (COG) of unigenes of *Acacia koa* annotated against the nr and TrEMBL databases**.** A total of 18,320 unigenes were classified into 26 functional categories. Some of the unigenes were assigned to more than one category.

4.3.5. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification

The KEGG database provides systemic functional information of biochemical pathways and functions of gene products. From the 43,309 annotated unigenes, 12,646 unigenes were grouped into 208 KEGG biochemical pathways (Fig. 15). Major KEGG biochemical pathway

groups were metabolism (5,729 unigenes), genetic information processing (2,707 unigenes), environmental information and processing (507 unigenes), cellular processes (1,381 unigenes), and organismal system (1,306 unigenes). The largest metabolic pathway groups include carbohydrate metabolism (1,269 unigenes), nucleotide metabolism (1,100 unigenes), and amino acid metabolism (836 unigenes). The pathways related to genetic information processing involved folding, sorting and degradation (1,179 unigenes), translation (939 unigenes), and replication (306 unigenes). Biochemical pathways for cellular processes were most represented by pathways for cell growth and death (787 unigenes), and transport (487 unigenes).

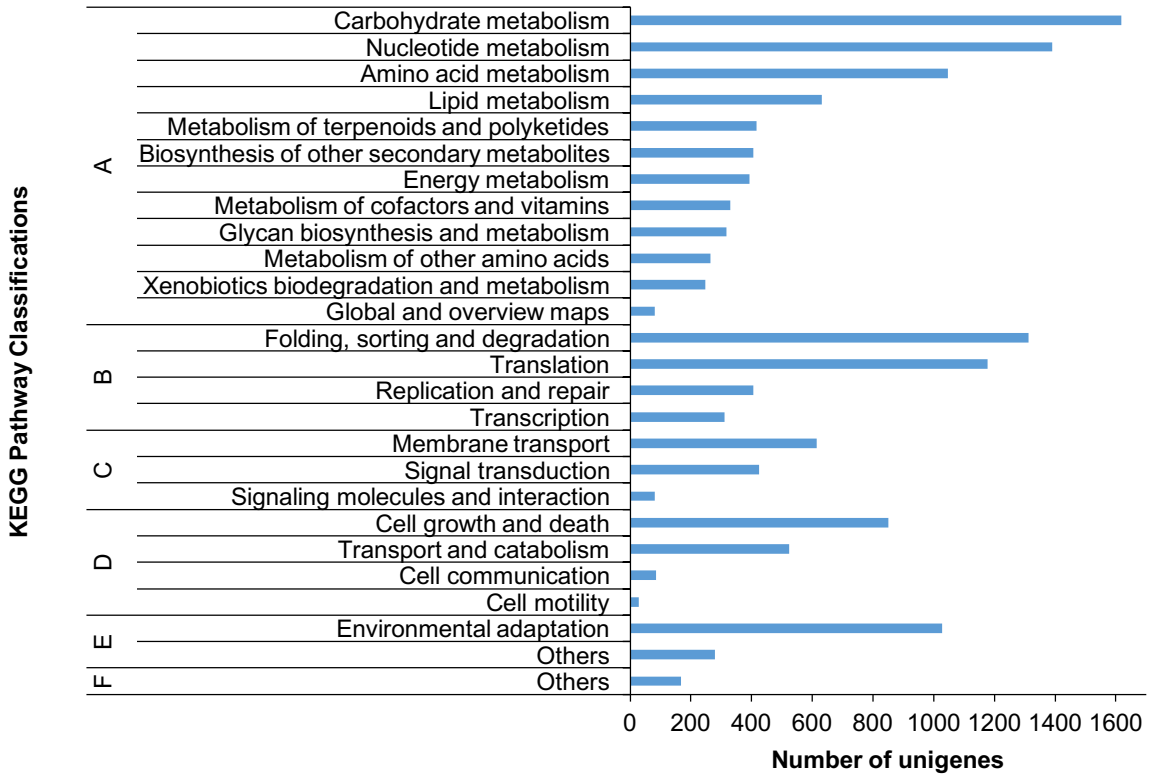

Figure 15. KEGG pathway classification of unigenes of *Acacia koa* annotated against the nr and TrEMBL databases. A total of 12,646 unigenes were grouped into 208 KEGG biochemical pathways. Categories: A = metabolism, B = genetic information processing, C = environmental information processing, D = cellular processes, E = organismal systems, F = others.

4.3.6. Identification of genes involved in the phenylpropanoid biosynthesis and plant immune response pathways

Through the KEGG pathway analysis, a total of 149 orthologs for ten enzymes involved in phenylpropanoid biosynthesis were identified, namely phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), *p*-coumarate:CoA ligase (4CL), cinnamoyl CoA reductase

(CCR), cinnamyl alcohol dehydrogenase (CAD), *p*-coumarate 3-hydroxylase (C3H), hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), caffeic acid O-methyltransferase (COMT), ferulate 5-hydroxylase (F5H), and caffeoyl CoA 3-O-methyltransferase (CCoAOMT). BLASTX analysis confirmed the functions of the unigenes in these ortholog groups. Also, the complete coding sequences (CDSs) were identified for 20 unigenes representing isoforms of the monolignol biosynthesis enzymes. They all had sequence similarities of > 70% with other legume species (Table S1).

Among 1,306 unigenes in the organismal system category, 947 were classified into 34 KEGG ortholog groups in the plant immune response pathways (KEGG name: plant-pathogen interaction pathways), including leucine-rich-repeat (LRR)-receptor kinases, disease resistance (R) proteins, WRKY transcription factors, chitin elicitor receptor kinases, and pathogenesis-related (PR) proteins. BLASTX analysis confirmed the functions of some of the unigenes in these ortholog groups. Complete CDSs were also identified for 13 unigenes, representing 8 ortholog groups, namely cyclic nucleotide-gated channel (CNGC), nitric oxidase-associated protein 1 (NOA1), respiratory burst oxidase homolog (Rboh), chitin elicitor-binding protein (CEBiP), chitin elicitor-receptor kinase 1 (CERK1), pathogenesis-related protein 1 (PR1), WRKY29, and resistance signaling protein RAR1 (Table S1). All except WRKY29 showed > 50% sequence identity. The CDSs of the unigenes were deposited at NCBI Transcriptome Shotgun Assembly (TSA) under the accession number GBYE00000000.

4.3.7. Putative simple sequence repeats (SSRs)

For development of new molecular markers, the 43,309 annotated unigenes were used to identify potential simple sequence repeats (SSRs). With the MISA Perl script, di- to hexa-nucleotides with a minimum of four repetitions were searched, and 13,109 unigenes containing a total of 20,755 putative SSRs were identified. Among them, 4,731 unigenes had more than one SSR (Table 5). Of those, 2,699 had SSRs in compound formation, having two or more consecutive SSRs interrupted by less than 100 bp. In total, 111 different motifs were detected. Di-nucleotide repeats except CG/CG (0.35%) were the most abundant (71.95%), and tri-nucleotide was the second abundant (26.95%; Table 6). The dominant repeat motif was AG/CT (45.28%), followed by AT/AT (13.72%), AC/GT (12.45), and AAG/CTT (8.78%; Fig. 16).

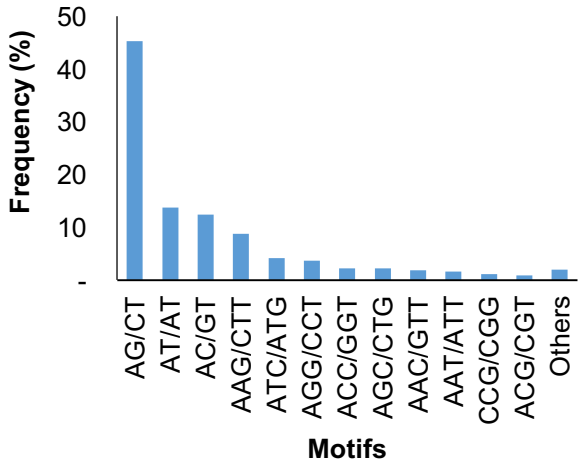

Figure 16. Frequency distribution of simple short repeats (SSRs) based on motif sequence types. A total of 111 motifs were identified from the *Acacia koa* transcriptome data.

Table 5. Summary of simple sequence repeat (SSR) searching results

Searching item	Numbers
Total number of sequences examined	43,309
Total size of examined sequences (bp)	41,338,838
Total number of identified SSRs	20,755
Number of SSR containing sequences	13,109
Number of sequences containing more than 1 SSR	4,731
Number of SSRs present in compound formation	2,699
Di- nucleotide	14,901
Tri- nucleotide	5,594
Tetra-nucleotide	153
Penta-nucleotide	35
Hexa-nucleotide	72

Table 6. Length distribution of simple sequence repeats (SSRs) based on the number of repeat units

Number of repeat unit	Di-	Tri-	Tetra-	Penta-	Hexa-
4	11220	3885	131	28	56
5	1927	1143	14	7	16
6	705	411	6	0	0
7	399	127	2	0	0
8	247	21	0	0	0
9	172	7	0	0	0
10	148	0	0	0	0
11	72	0	0	0	0
12	10	0	0	0	0
13	1	0	0	0	0
Total	**14901**	**5594**	**153**	**35**	**72**

4.4 *Discussion*

4.4.1. Transcriptome sequencing and assembly

Despite numerous studies on genomes of various legume species, only a limited number of nucleotide or protein sequences of *A. koa* have been reported, and almost no genomic information is available in public databases. As a majestic timber tree, *A. koa* could be a rich source of genes for tree improvement programs. Thus, the objective of this study was to produce a global overview of the whole transcriptome of *A. koa*. After comparing with the five databases and filtering out all the mostly small, unannotated sequences, a total of 43,309 unigenes were identified in this study. A large proportion of smaller unigenes obtained through Illumina sequencing may be due to the allotetrapolyploid genome of *A koa* (2*n* = 52); homeologous or paralogous gene copies can be distinct yet highly similar, possibly causing incomplete assembly (Duan et al. 2012; Gruenheit et al. 2012; Nakasugi et al. 2014). Both the average length and the N50 length obtained from *A. koa* in the present study were greater than those obtained from other related diploid legume species, such as *Acacia auriculifomis* (496 and 949 bp), *Acacia mangium* (498 and 938 bp; Wong et al. 2011), and *Cicer arietinum* (523 and 900 bp; Garg et al. 2011). Also, the total number and cumulative length of unigenes of *A. koa* were more than twice of those of *A. auriculiformis* (42,217 unigenes and 21.02 Mb) and *A. mangiu*m (35,759 unigenes and 17.84 Mb; Wong et al. 2011). These differences may be also due to their ploidy levels, as *A. auriculiformis* and *A. mangium* are both diploid (2*n* = 26), in addition to the use of different assembly software in those studies.

4.4.2. Possible genes involved in the plant immunity in *A. koa*

Acacia koa forests in Hawai'i are heavily infested with wilt and dieback caused by *F. oxysporum f. sp. koae* (Gardner 1980; Whitesell 1990; Wilkinson and Elvevitch 2003). As certain *A. koa* populations are known to be more resistant than others (Dudley et al. 2008), a genetic predisposition appears to be one of the key factors for susceptibility and resistance to this disease. Therefore, analysis of genes that may be associated with disease resistance is crucial. In the present study, the unigenes were screened to identify genes involved in the plant immunity. The KEGG analysis detected 34 ortholog groups in the plant immune response pathways. There are two types of plant immune system, namely microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) and effector-triggered immunity (ETI), as described in Chapter 2.

Many unigenes involved in MTI were detected, including 49 unigenes encoding orthologs of CNGC, 1 unigene encoding an ortholog of NOA, and 11 unigenes encoding orthologs of Rboh. Among those, complete CDSs were identified for four CNGC genes, one NOA gene, and three Rboh genes (Table S1). Previous studies have shown that these proteins play an important role in plant defense. According to Clough et al. (2000), disrupting CNGCs resulted in an impaired hypersensitive response (HR) in *Arabidopsis*. Also, silencing NOA1 in *Nicotiana benthamiana* reduced NO production and increased susceptibility to a fungal pathogen *Colletotrichum lagenarium* (Kato et al. 2008). In *Arabidopsis* and *N. benthamiana, rboh* mutants were susceptible to various fungal pathogens (Reviewed by Marino et al. 2012). CNGC, NOA and Rboh, thus, could be key components in resistance to fungal pathogens in *A. koa* as well.

Plants can also perceive fungal pathogens by recognizing chitin of fungal cell walls with CEBiP and CERK1 (Wan et al. 2008). The KEGG analysis detected 4 orthologs of CEBiP and 41 orthologs of CEPK1. Among them, two unigenes had complete CDSs for CEBiP and CEPK1-like protein (Table S1). Recent studies have shown their importance in disease resistance; *cerk1* mutants in *Arabidopsis* (Miya et al. 2007; Wan et al. 2008) and *cebip* mutants in rice (Kishimoto et al. 2010; Liu et al. 2012) had compromised disease resistance against fungal pathogens *Alternaria brassicicola* and *Magnaporthe oryzae,* respectively. Kishimoto et al. (2010) constructed a chimeric receptor kinase containing CEBiP and kinase regions of another receptor-like kinase with ability to induce HR-like response and observed that the chimeric receptor kinase increased responses to chitin elicitor and conferred resistance against *M. oryzae*. In addition to CEBiP and CERK1, all the four major disease-related classes of chitinase in plants were identified from the transcriptome of *A. koa* (Hamel et al. 1997; Kasprzewska 2003). Rushanaedy et al. (2012) observed higher expression of chitinases in *A. koa* families resistant to *F. oxysporum* compared to susceptible families upon infection by virulent strains of *F. oxysporum*. Chitinases play an important role in plant defense response as they degrade the fungal cell wall to limit fungal invasion.

Besides fungal pathogens, bacterial pathogens, such as *Xylella fastidiosa,* are found to cause diseases in *Acacia* species (Janse and Obradovic 2010). Flagellin proteins (flg22) and elongation factor Tu (EF-Tu) of bacterial pathogens are recognized by LRR-RLKs named flagellin-sensing2 (FLS2) and elongation factor-Tu receptor (EFR), respectively. Both receptors form heterodimers with brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1) upon the recognition of the MAMPs (Sun et al. 2013). FLS2-BAK1 and EFR-BAK1 then induce

mitogen-activated protein kinase (MAPK) signaling cascades to express defense-related genes. The transcriptome analysis of *A. koa* detected unigenes orthologous to FLS2 (143 unigenes), EFR (46 unigenes), and BAK1 (58 unigenes; data not shown). In *Arabidopsis*, *fls2* and *efr* mutants were insensitive to flg22 and EF-Tu, respectively, inducing susceptibility to bacterial pathogens such as *Agrobacterium tumefaciens* and *Pseudomonas syringae* (Gómez-Gómez and Boller, 2000; Zipfel et al. 2004; Zipfel et al. 2006), and *bak1* mutants were partially insensitive to both flg22 and EF-Tu (Chinchilla et al. 2007). Therefore, these genes may also be essential in pathogen recognition in other plants, including *A. koa*.

Defense-related genes that are known to be induced following the recognition of the MAMPs were identified, including 6 orthologs and 1 complete CDS of WRKY29 and 11 orthologs and 1 complete CDS of PR1. Although their biological functions are still obscure, their roles in disease resistance are critical. For example, resistance to a fungal pathogen *Botrytis cinerea* was conferred by overexpression of WRKY29 in *Arabidopsis* (Asai et al. 2002) and also by overexpression of PR1 in tobacco (Kiba et al. 2007). PR1 also can be used as a marker for HR-mediated defense response (Lu et al. 2011).

Through the KEGG analysis, genes coding for proteins homologous to various resistance proteins (R proteins) involved in ETI were identified, including RPM1 (100 unigenes), RPS2 (77 unigenes), and RPS5 (14 unigenes), as well as resistance signaling protein RAR1 (1 unigene). These R proteins may participate in the recognition of effector molecules secreted by bacterial or fungal pathogens during infection, and in the initiation of defense signaling networks inducing HR (Hammond-Kosack et al. 2007). RPM1, RPS2, and RPS5 have been identified in *Arabidposis,* and they mediate the recognition of specific avirulence gene products encoded by pathogens, leading to defense signaling pathways (Boyes et al. 1998; Axtell et al. 2003; Qi et al. 2012). *Arabidopsis* with defective RAR1 exhibited decreased disease resistance against pathogens, and RAR1 was found to determine the extent of oxidative burst at infection sites, resulting in HR and pathogen containment, thereby acting as a rate-limiting regulator of early R gene-triggered defense (Muskett et al. 2002). The ortholog groups in the plant immune response pathways detected in *A. koa* in the present study, including R proteins, CNGC, CEBiP, CERK1, FLS2, and EFR, may be a key to confer resistance to diseases like *Fusarium* wilt in *A. koa.*

4.4.3. Genes involved in the phenylpropanoid biosynthesis pathway in *A. koa*

In addition to genes involved in plant immune system, this study aimed to identify genes in the phenylpropanoid biosynthesis pathway in *A. koa* because the pathway is associated with disease resistance. The phenylpropanoid metabolism generates a wide variety of secondary metabolites associated with pathogen resistance, such as lignin and flavonoids (Fig. 17). In this study, 21 ortholog groups in the phenylpropanoid biosynthesis pathway were identified from the KEGG pathway analysis. Of those, unigenes encoding orthologs of the enzymes involved in the upstream of the pathway were detected, including 14 orthologs of PAL, 3 orthologs of C4H, and 11 orthologs of 4CL. The induction of the phenylpropanoid biosynthesis in response to disease infection has been observed in various plants. In wheat, infection with a fungal disease Karnal bunt significantly elevated the activity of PAL and the lignin content in resistant genotype (Purwar et al. 2012). Also, in *M. truncatula,* the expression levels of the genes for C4H and 4CL were significantly elevated following the infection by a fungal pathogen *Phoma medicaginis* (Kamphuis et al. 2012). These reports show that PAL, C4H, and 4CL are involved in disease resistance in various plants. It is likely that these enzymes may also play an important role in disease resistance in *A. koa*.

Also, the monolignol biosynthesis pathway, a branch of the phenylpropanoid biosynthesis pathway, is also associated with plant defense as well as growth and development. All the ten enzymes for monolignol biosynthesis (Fig. 17) were identified in this study. Monolignols, which consist of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, are the building blocks of lignin. Lignin is one of the major components of the secondary cell wall, so it is important as plant physical defense (Plomion et al. 2001). Several studies have shown the increased lignin content in response to infection by fungal pathogens in various plants (Kamphuis et al. 2012; Purwar et al. 2012), so regulations of the monolignol biosynthesis enzymes may be important mechanisms of disease resistance. In addition, because the monolignol biosynthesis pathway is part of the phenylpropanoid pathway, regulations of the monolignol biosynthesis enzymes can affect other metabolite production. For example, repression of a *HCT* gene in *A. thaliana* resulted in accumulation of flavonoids, such as anthocyanin (Besseau et al. 2007).

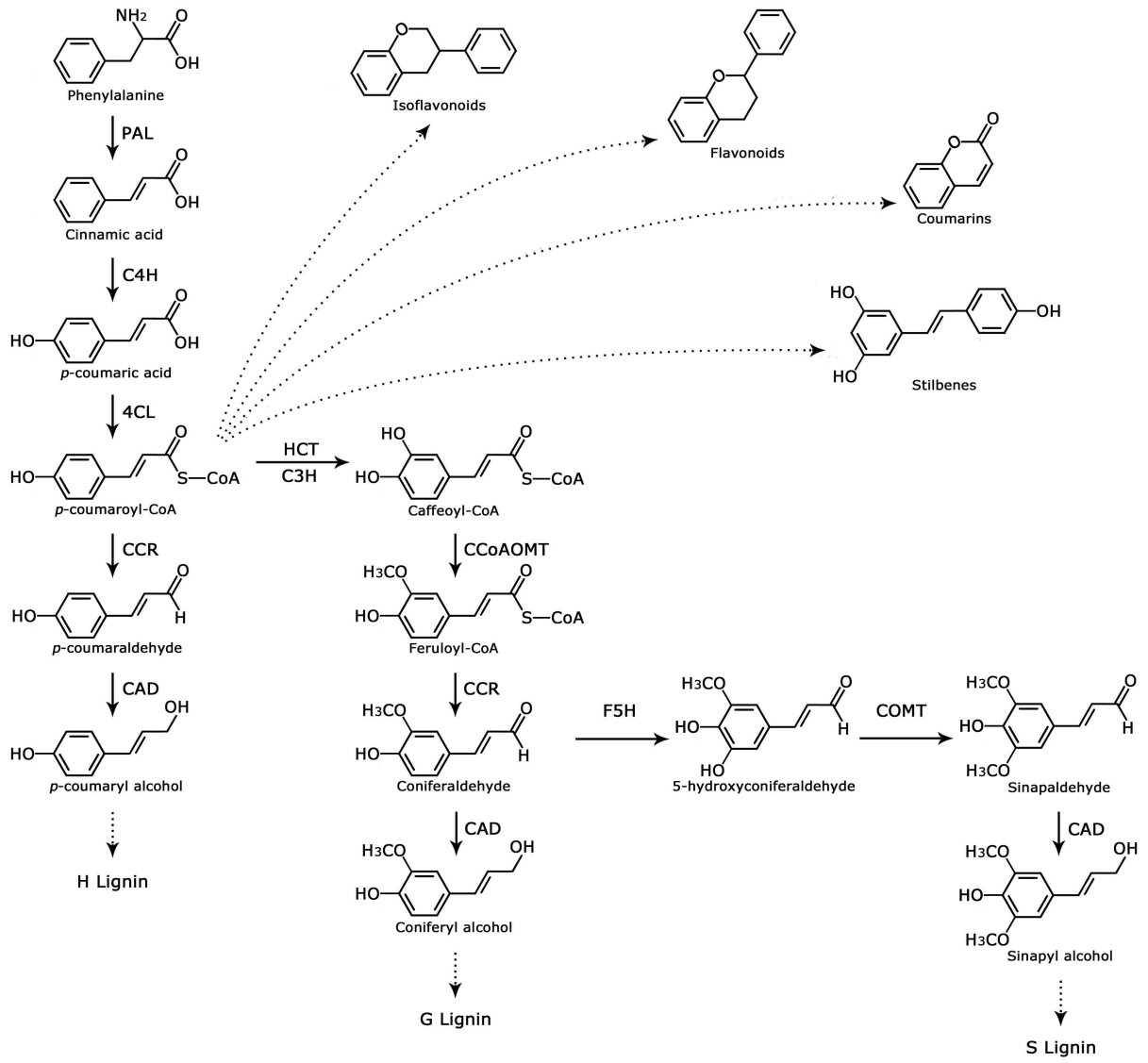

Figure 17. Monolignol biosynthesis pathway. Because of the variety of isoenzymes and kinetic properties, alternative routes through the metabolic pathway may exist. Dashed arrows represent multiple reaction steps. Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydoxylase; 4CL, *p*-coumarate:CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; C3H, *p*-coumarate 3-hydroxylase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; COMT, caffeic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase.

In the present study, 149 unigenes were assigned as orthologs of the enzymes involved in the phenylpropanoid biosynthesis pathway (Fig. 17). However, there are many closely related superfamily members ("like" proteins), and some of them may be unrelated to the phenylpropanoid biosynthesis pathway, so unigenes with < 50% homology with *A. thaliana* genes were excluded besides unigenes without important conserved amino acid motifs

identified in previous studies (McKie et al. 1993; Wanner et al. 1995; Schuler 1996; Joshi et al. 1998; Ehlting et al. 2001; Lynch et al. 2002; Zubieta et al. 2002; Larsen 2004). Complete CDSs of genes for all the ten enzymes involved in monolignol biosynthesis were identified (Table S1). There may exist more isoforms in *A. koa* because conserved motifs could not be determined in some of the unigenes due to incomplete assembly. Future studies of *A. koa* involving significant variations in disease resistance will determine the level of expressions of key phenylpropanoid biosynthesis genes that result in specific phenotypes. Therefore, determining the expression levels of those key genes will be useful for selection for improved disease resistance.

4.4.4. Putative simple sequence repeat (SSR) molecular markers

Next-generation sequencing (NGS) is a rapid and effective approach to identify SSR molecular markers in non-model organisms without known genomic sequences. The traditional approach to develop SSR molecular markers involves fragmentation of DNA, construction of genomic DNA libraries in *Escherichia coli*, PCR amplification, and sequencing of the amplified fragments (Song et al. 2005; Sahu et al. 2012). These steps are time-consuming and labor-intensive. Using NGS technology and bioinformatics, identification of numerous SSRs from the sequence data can be rapid and cost-effective. Previously, through the traditional approach, Fredua-Agyeman et al. (2008) analyzed 96 sequences and developed 31 primer pairs that targeted microsatellite loci in *A. koa*. Some of these primers successfully identified polymorphic loci and were also used to measure genetic diversity in *A. koa* (Adamski et al. 2012); yet, only limited number of genetic markers exists in *A. koa*, so the identification of more SSRs with NGS technology will be useful.

In the present study, 13,109 unigenes containing a total of 20,755 putative SSRs were predicted. In *A. koa*, dinucleotide repeats were the predominant motif as in many other plants, such as *A. thaliana*, *Arachis hypogaea*, *Brassica napus*, *Beta vulgaris*, *Brassica oleracea*, *G. max*, *Vitis vinifera*, and *Sesamum indicum* (Kumpatla and Mukhopadhyay 2005; Wei et al. 2014). The frequency of SSR motifs in *A. koa* obtained in this study was consistent with that of other plant species. The AG/CT repeat (45.28%) was the most abundant dinucleotide motif group, and the CG/CG repeat (0.35%) was the smallest dinucleotide motif group in *A. koa* just as in various species studied by Jayashree et al. (2006) and Kumpatla and Mukhopadhyay (2005). The AAG/CTT repeat (8.78%) was the predominant trinucleotide motif in *A. koa*, and it was also predominant in other plants, including three legume species, *G. max*, *M. truncatula*, and *Lotus japonicus* (Kumpatla 2005; Jayashree et al. 2006). The results in this study provide a

substantial number of SSRs; in future studies, it may be possible to identify SSR loci linked to genes associated with disease resistance.

4.4.5. Conclusions

This is the first comprehensive transcriptome-wide analysis of *A. koa* using NGS technology. Illumina sequencing and Trinity *de novo* assembly generated 85,533 unigenes, and 43,309 of them were successfully annotated. The KEGG analysis identified 34 gene ortholog groups involved in the plant immune response pathways and 21 involved in the phenylpropanoid pathway, which is highly associated with disease resistance. Further characterization of these genes will contribute to a deeper understanding of disease resistance and wood quality in *A. koa*. These genes may be useful as molecular markers for screening of *A. koa* seedlings with high disease resistance and wood quality. In addition, a significant number of potential SSR markers were predicted from the *A. koa* transcriptome data. These results will be a valuable resource for future genetic studies and improvement programs of *A. koa*.

CHAPTER 5
RESULTS FOR SPECIFIC OBJECTIVE 2

Thigmomorphogenesis: morphological, biochemical changes, and transcriptional level changes in response to mechanical stress in *Acacia koa*

5.1. *Introduction*

Mechanical perturbations, such as predation, rain, and wind, are common environmental stresses for plants. Because plants are sessile and cannot travel to a new habitat on their own, the ability to sense, respond, and adapt to such environmental stresses is important for their survival. Responses can be rapid and apparent in some plants with specialized sensory cells. For example, *Dionaea muscipula* closes bi-lobed leaves as soon as an insect lands on the ventral leaf surface, which has 'trigger hairs' with sensory cells at the base (Volkov et al. 2008). Another plant species, *Mimosa pudica,* has specialized pulvinar motor tissues (Visnovitz et al. 2007) and the leaflets fold up quickly after touch stimulation (Temmei et al. 2005; Volkov et al. 2010); this behavior may protect the leaves from insect predation or from damage during rain or windstorms (Koller 2011).

Plants without specialized sensory cells also respond to mechanical stimuli. Morphological responses in such plants may not be noticeable immediately, but their growth and development are slowly altered over time in response to prolonged mechanical stimulation. Such physiological and developmental responses of plants to mechanical stimuli are called thigmomorphogenesis (Jaffe 1973). For example, plants repeatedly swayed by wind typically respond by growing shorter and stockier as to protect themselves from the stimuli (Jacobs 1954). In experimental studies, wind-induced motion of plants has been artificially generated under controlled laboratory conditions by touching or bending stems back and forth (Mitchell et al. 1975, Jaffe 1979, Biro et al. 1980, Biddington and Dearman 1985). Such mechanical perturbations commonly result in decreased elongation and increased thickness, and plants respond to wind in the same way (Telewski and Jaffe 1986*a*; Braam and Davis 1990; Lee et al. 2005). These typical thigmomorphogenetic responses are observed in various plants, including *Arabidopsis thaliana*, *Carcica papaya*, and woody plants, such as *Populus* plants, *Abies fraseri,* and *Pinus taeda* (Jacobs 1953; Biro et al. 1980; Telewski and Jaffe 1986*a* 1986*b*; Pruyn et al. 2000; Lee et al. 2005; Porter et al. 2009). Similar morphological changes have been also observed to other mechanical stimuli, such as rubbing and brushing of the stems, in various plants, including *Arabidopsis, Bryonia dioica, Phaseolus vulgaris,* and *Solanum lycopersicum* (Jaffe 1973; Saidi et al. 2009; Paul-Victor and Rowe 2011).

Interestingly, some plants do not show typical thigmomorphogenetic responses, and also show other diverse responses. For example, *Cucurbita pepo*, *Pisum sativum*, *Triticum aestivum* display little to no changes in elongation after mechanical perturbations (Jaffe 1973), and the stem diameter, instead of increasing as in few other thigmomorphogenetic plants, decreased in *Brassica oleracea* (Biddington and Dearman 1985). Different morphological changes have been observed in different plant species, such as a delay in flowering and an increase in number of rosette paraclades in *Arabidopsis* (Johnson et al. 1998), tissue outgrowths in petioles, and decreased anthocyanin contents in *C. papaya* (Porter et al. 2009).

Although morphological changes occur gradually, genes induced by mechanical stimuli are immediately detectable (Braam and Davis 1990; Lee et al. 2005; Porter et al. 2009). In *Arabidopsis,* approximately 2.5% of all genes were upregulated at least two-fold within 30 min following mechanical stimulation (Lee et al. 2005). These induced genes encode proteins with diverse functions, such as calcium-binding proteins, cell-wall-modifying enzymes, kinases, transcription factors, and disease resistance proteins, indicating complex molecular mechanisms involved in the perception of mechanical stimulation (Lee et al. 2005). Because some thigmomorphogenetic responses appear to be species-specific, different pathways may be involved in the perception of mechanical stimulation in different plants; therefore, it is important to study thigmomorphogenesis in various plants.

This study presents morphological and biochemical changes and levels of gene transcription in response to mechanical stimulation in *Acacia koa* (koa), a leguminous timber tree endemic to the Hawaiian Islands. *Acacia koa* serves as a vital economical resource for the Hawaiian Islands as its timber is a highly priced commodity due to the beautiful texture, hardiness, and carving quality of the wood. With a current market value of up to $125 per board foot, the gross value of *A. koa* timber and the wood products produced is estimated to be in the range of $20-$30 million annually (Yanagida et al. 2004; Baker et al. 2009). To grow *A. koa* successfully under various conditions of mechanical stimulation, such as heavy rain, predation, and wind, understanding how mechanical stimulation influences the plant growth and development is crucial. This study of morphological, physiological, and molecular aspects of thigmomorphogenesis may contribute towards *A. koa* improvement programs and further understanding of this phenomenon.

5.2. *Materials and Methods*

5.2.1. Plant growth and treatment

Seeds of *A. koa* were obtained from the Maunawili Research Station of Hawai'i Agriculture Research Center (HARC), Kailua, Hawai'i. Seeds were immersed in concentrated sulfuric acid for 10 min for scarification, rinsed with sterilized water six times, and incubated in petri dishes with wet paper towels at 28 ^{o}C till they germinated in 3-5 days (Rushanaedy et al. 2012). The resulting germinated seedlings were then planted into 4-inch pots containing a vermiculite-perlite mixture pre-wetted with Hoagland's solution. The seedlings were grown in a controlled growth room at 25 °C $\pm$ 2 °C with a 16/8-h light/dark photoperiod with an irradiance of 30 µmol s^{-1} m^{-1} for a month. Plants were watered with Hoagland's solution once in two days. For a mechanical stress, stems were gently bent from its position at 90º with the ground level to a position at approximately 60º with the ground level in each of all the four cardinal directions manually. Plants were mechanically treated daily starting from a week after seedlings were transferred to pots. The seedlings in the control group were maintained similarly, except that they were not given any mechanical stress.

5.2.2. Phenotypic measurements and microscopy

Plant heights, stem diameters, and fresh and dry weights of stems, roots, and leaves of 2- and 6-month-old koa seedlings were quantified. Plant heights were measured from the base of the stem to the shoot tip. Stem diameters were measured at the stem base using a 5-inch digital caliper. Each of the stressed and unstressed groups represented 39 individual 2-month-old seedlings and 15 individual 6-month-old seedlings. For microscopic analysis, 2-month-old koa seedlings were used; 30-µm cross sections of stems at 5 mm below the top of the hypocotyls were made using a VT1000S vibratome (Leica Biosystems Inc., IL, USA), and the cross sections were observed under an inverted microscope DMIL (Leica Biosystems Inc.). The number and area of the xylem cells in three radial files were manually quantified using the images obtained from the microscope. Three plants were randomly selected from each of the stressed and unstressed groups for this analysis. Statistical significance for change in the stressed plants relative to unstressed plants was determined by comparing the average of the two groups through Student's two-tailed t-test with a cutoff *p*-value of 0.05. Similarly, the stem cross sections of 3-month old unstressed plants were analyzed and compared with those of the 2-month old stressed plants.

5.2.3. Anthocyanin, chlorophyll, and lignin quantification

After removal of leaves, stem tissue of 2-month-old koa seedlings was frozen in liquid nitrogen and ground using a mortar and pestle for tissue homogenization. For every 0.1g of the stem tissue, 1.0 mL of EtOH containing 1% HCl was used to extract anthocyanin. After mixing the tissue and the solution, each sample was incubated at 4 ºC overnight and then centrifuged for 10 min at 15,000 rpm. Absorbance of the supernatant was quantified at 529 nm and 650 nm using a UV-visible spectrophotometer, UV-1600PC (VWR, PA, USA). To calculate the corrected anthocyanin absorbance, Eq. (1) was used.

$$\text{Anthocyanin absorbance} = A_{529} - (0.228)\,(A_{650}) \quad (1)$$

The anthocyanin concentration was calculated using the Beer-Lambert expression, $A = \varepsilon c l$, where A is absorption, ε is a molar absorbance coefficient, c is concentration, and l is path length. The molar absorbance coefficient ε of anthocyanin at 529 nm was assumed to be 30,000 L mol^{-1} cm^{-1} (Sims and Gamon 2002). Similarly, chlorophyll was extracted using 1.0 mL of 80% acetone per 0.1 g of sample. Absorbance of the supernatant was quantified at 647 nm and 664 nm, and Eqs. (1-4) were used to calculate the concentrations of chlorophyll *a*, chlorophyll *b*, and total chlorophyll (Porra 2002). For quantification of anthocyanins and chlorophylls, 15 individual plants were analyzed for each of the stressed and unstressed groups.

$$\text{Chlorophyll } a \text{ (µg/mL)} = 12.25\,(A_{664}) - 2.55\,(A_{647}) \quad (2)$$

$$\text{Chlorophyll } b \text{ (µg/mL)} = 20.31\,(A_{647}) - 4.91\,(A_{664}) \quad (3)$$

$$\text{Total chlorophyll (µg/mL)} = 17.76\,(A_{647}) + 7.34\,(A_{664}) \quad (4)$$

Stem lignin analysis was conducted by the Agricultural Diagnostic Services Center, University of Hawai'i at Mānoa through an ANKOM 200 Automated Fiber Analyzer (ANKOM Technology, Macedon, NY) as described by the manufacturer's protocol for lignin quantification in Method 8 (https://www.ankom.com/). Three biological replicates were analyzed, and each replicate represented a pool of 10 hypocotyls. For each analysis, statistical significance for change in the stressed plants compared to unstressed plants was determined by comparing the average of the two groups through Student's two-tailed t-test with a cutoff *p*-value of 0.05.

5.2.4. Plant treatment and RNA extraction

For mechanical stress treatment, starting from one week after seedlings were transferred to pots, stems were manually and gently bent from its position at 90º with the ground level to a position at approximately 60º with the ground level in each of all the four cardinal directions manually. Stems were harvested via flash-freezing in liquid nitrogen after 10, 30, and

60 min following the mechanical stress treatment. To extract total RNA, leaves were removed from the stems, and stems were ground to a fine powder in liquid nitrogen with a mortar and a pestle that were previously baked for 6 h at 300 °C prior to use. Total RNA was extracted with the modified method using Qiagen RNeasy® Plant Kit (Valencia, CA, USA) and Fruit-mate (Takara, Japan) as described by Ishihara et al. (2016). RNA was treated with TURBO DNA-free Kit (Ambion, CA, USA) to remove genomic DNA contamination. The quantity and quality of the RNA were assessed at wavelengths of 230, 260, and 280 nm using a NanoDrop ND-1000 (NanoDrop Technologies, DE, USA). The integrity of RNA was confirmed with RNA Integrity Numbers (RIN) of ≥ 9 obtained through an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA).

5.2.5. Microarray analysis

Relative gene expression levels of the stressed plants relative to the unstressed control were determined through a microarray analysis. An equal amount of extracted RNA of the *A. koa* seedlings harvested at 10, 30, and 60 min following the mechanical stress treatment was pooled for the analysis. The RNA extracted from the seedlings without the mechanical stress treatment was used as the unstressed control. A 4x72K microarray analysis was performed by the Roy J. Carver Center for Genomics at the University of Iowa to compare gene expression levels of 2 biological replicates of 30 stressed plants (10 plants/time point) and 2 biological replicates of 20 unstressed control plants. From the previous study of transcriptome analysis (Ishihara et al. 2015), 4,000 cDNA sequences longer than 360 bp were selected on the basis of similarities with genes that might be related to plant defense and growth. Six 60-bp probes per sequence were designed by the Carver Center for Genomics; each probe had three replicates in a DNA chip. The designs of probes and microarray were deposited into GEO (Record No. GSE73889). The array was scanned using a NimbleGen MS 200 Microarray Scanner (Roche NimbleGen, Inc.). The NimbleScan software v.2.6 (Roche NimbleGen, Inc.) extracted raw intensities from the images generated by the scanner, which were corrected for background noise and normalized between arrays using a Robust Multichip Average (RMA) algorithm included in the NimbleScan software. The normalized probe intensity values were averaged to give a single intensity value per transcript and per sample. Statistical significance for change in the stressed plants relative to unstressed plants was determined by comparing the average of the two groups through Student's two-tailed t-test with a cutoff *p*-value of 0.05.

5.2.6. Confirmation of microarray data by NanoString nCounter and qRT-PCR

To verify if the sequences shown to be upregulated in the microarray analysis were truly upregulated, the NanoString nCounter System, a digital multiplexed gene-expression analysis, was used at NanoString Technologies, Seattle WA. Briefly, total RNA extracted (100 ng) from seedlings at 10, 30, and 60 min following the stress treatment and from seedlings without the stress treatment was prepared. Three biological replicates of RNA from ten stressed plants for each time point were compared with three biological replicates of RNA from ten unstressed plants. RNA was directly hybridized with gene-specific color-coded probes, and data collection was carried out in the nCounter Digital Analyzer as described by the manufacturer (NanoString Technologies). The NanoString Codeset was designed and synthesized by NanoString Technologies. It included 3 reference genes, elongation factor-1 alpha (*ef1α*), β-actin, and ubiquitin-5; six positive-control and eight negative-control probes were also added to each reaction to normalize the results. The data analysis was performed through nSolver Analysis Software (NanoString Technologies) using the default settings. Statistical significance for increase in gene expression levels in the stressed plants compared to unstressed plants was determined using Student's one-tailed t-test with a cutoff *p*-value of 0.05.

The nCounter results were further confirmed through quantitative real-time PCR (qRT-PCR) analysis of five genes. Total RNA extracted prior to the mechanical stress treatment and after 10, 30, and 60 min following the mechanical stress treatment was treated with TURBO DNAfree Kit (Invitrogen, Carlsbad, CA) to remove any genomic DNA contamination. First-strand cDNA was synthesized from 2 µg of DNase-treated RNA using M-MLV Reverse Transcriptase (Promega, WI, USA) with random hexamers according to the manufacturer's instructions. The qRT-PCR was performed using a 10 µL PCR reaction consisting of 0.25 µL forward primer (10 µM), 0.25 µL reverse primer (10 µM), 5 µL iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA), and 1 µL of first strand cDNA. Reaction conditions were 95 °C for 3 min, 35-40 cycles of 95 °C for 15 s, annealing for 30 s, and 72 °C for 30 s, followed by melting curve analysis of the amplicon to confirm the specificities of primers. The annealing temperature was at 54 °C for all the tested genes, except that 58 °C was used for the *RING* gene. Each assay consisted of three biological and three technical replicates and was performed using StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR protocol produced a PCR efficiency of 90% to 110% for each primer set. The primer sequences used for this study are listed in Table S2.

To select internal reference genes for relative quantification of target gene expressions, six reference candidate genes: *ef1α*, β-actin, 18S rRNA, 5.8S rRNA, ubiquitin-5, and tubulin-1 were tested on first strand cDNA samples from the *A. koa* stem with the primer sequences as described in Negi et al. (2011). The qRT-PCR protocol was performed as described previously, with the annealing temperature at 58 °C. The cycle threshold (Ct) values of the candidate genes were used to evaluate their expression stability by using NormFinder applet for MS Excel (Andersen et al. 2004). NormFinder allows the user to determine intra- and inter-group variances as well as the stability value of each candidate gene. One gene or a combination of two genes with the lowest stability value were selected as a reference control to normalize the expression levels at each time point: a combination of two genes, 18S rRNA and *ef1α*, for the time point at 10 min following the treatment, and 18S rRNA for the time points at 30 and 60 min following the treatment (Table 7). The fold changes of target gene expression levels relative to the unstressed control plants were determined using the selected reference genes for normalization through the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Statistical significance for increase in gene expression levels in the stressed plants compared to unstressed plants was determined using Student's one-tailed t-test with a cutoff *p*-value of 0.05.

Table 7. Stability values of candidate reference genes for quantitative real-time PCR (qRT-PCR) analysis determined by NormFinder

Gene names	10 min	30 min	60 min
Tubulin-1	0.473	0.432	0.397
ef1a	0.061	0.396	0.317
18S rRNA	0.048	0.123	0.132
Ubiquitin-5	0.073	0.322	0.257
5.8S rRNA	0.255	0.301	0.267
β-actin	0.165	0.535	0.520
The best combination*	0.039	0.157	0.153

*18S rRNA and ef1a for 10 min, 18S and 5.8S rRNA for 30 and 60 min

5.3. *Results*

5.3.1. Mechanical stress alters plant morphology by reducing stem elongation and increasing stem diameter in *A. koa*

Following two months of the daily mechanical perturbation, the stressed *A. koa* seedlings showed a decrease in stem elongation by 32.2% and an increase in hypocotyl diameter by 17.8% (Fig. 18A, C, and D). The mechanical stress also decreased the size of the leaflets by 19.8% and the dry weight of root by 44.6% (Fig. 18E and F). After six months of the treatment, plants showed greater developmental changes compared to the plants that were

stressed for two months, except for the root dry weight. The stressed plants were stunted by 60.9% and had a significantly increased hypocotyl diameter by 23.6% (Fig. 18B, D, and C). The leaflet size decreased approximately by 32.2% (Fig. 18E). Interestingly, the root dry weight decreased by 27.2% (Fig. 18F), but the percentage in decrease was greater after two months (44.6%) than after 6 months. There was no significant difference in dry weight of hypocotyls in spite of the difference in height (data not shown).

5.3.2. Mechanical stress increases anthocyanin and lignin but not chlorophyll

Mechanical stress increased the anthocyanin content in stems of the stressed seedlings by 91.0% following 2 months of the treatment (Fig. 19B). It was visually observable as well (Fig. 19A); the stressed seedlings exhibited anthocyanin accumulation in their stems with reddish color. The lignin composition in the stressed stems also increased by 32.3% (Fig. 19C). There was no significant difference in chlorophyll concentration (data not shown).

5.3.3. Mechanical stress promotes production of xylem cells

Microscopic analysis of hypocotyl cross sections showed an increase in the density of xylem cells (number of cells/10,000 μm^2) by 45.7% in the stressed seedlings compared to unstressed (Fig. 20A, B, and E). The increase in cell density was due to the reduction in the cell size, as the average size of a xylem cell was smaller in stressed plants by 32.4% (Fig. 20D). Regardless of the increase in the cell density following the mechanical stress, the ratio between the radii of the stem and the xylem did not change (data not shown). To determine if xylem cell size have correlation with stem diameter, stem cross sections of 2-month-old stressed seedlings were also compared with those of 3-month-old unstressed seedlings with similar stem diameters; the xylem cell density of 2-month-old stressed plants was 66.3% higher than 3-month-old unstressed plants whereas the cell size of the 2-month-old stressed plants was lower by 40.5% than 3-month-old unstressed plants (Table S3). The results showed no correlation between the stem diameter and the xylem cell size, and thigmomorphogenetic plants have higher cell density with smaller cells, while unstressed plants have lower cell density with bigger cells.

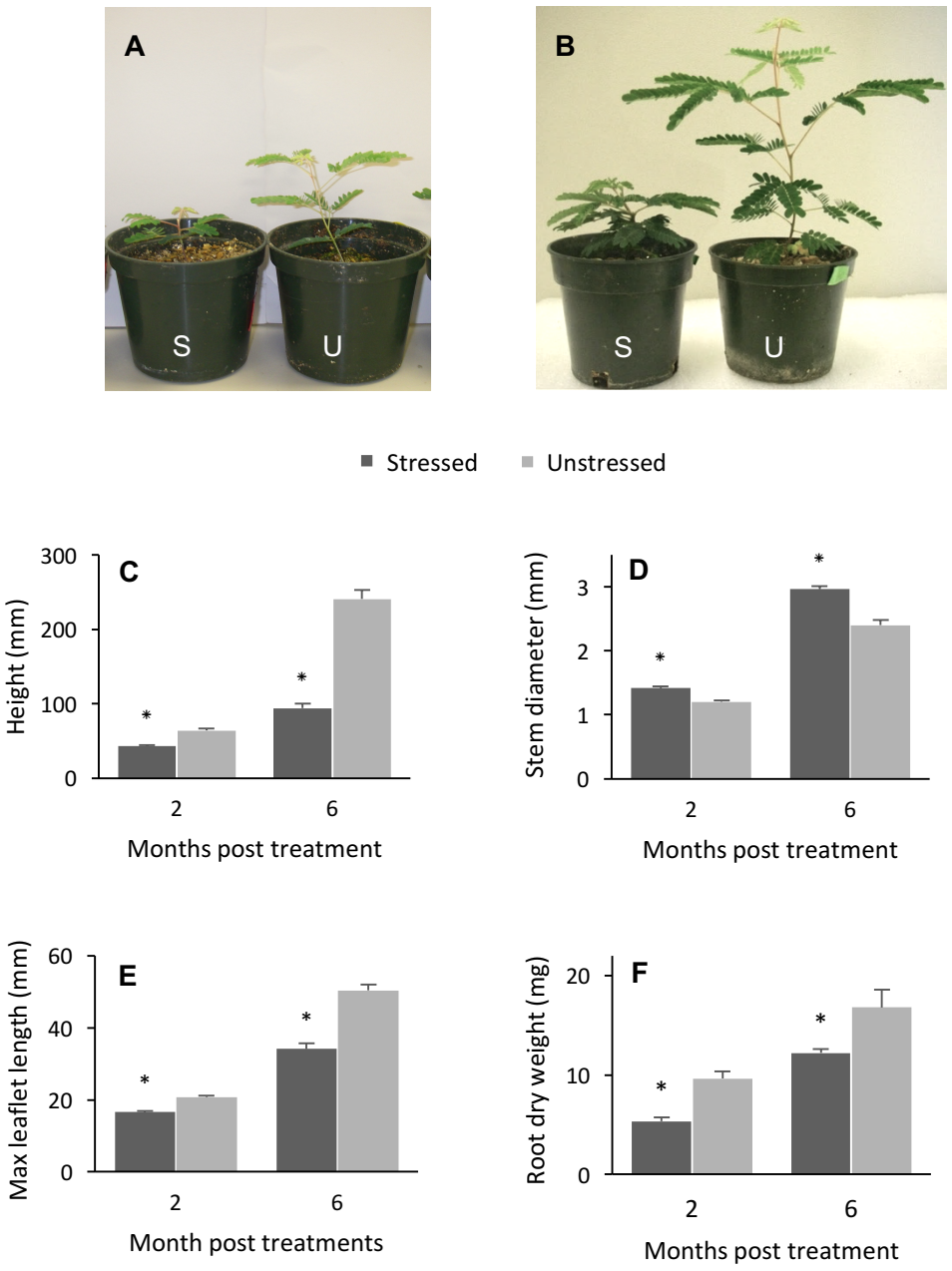

Figure 18. Phenotypical quantifications of *Acacia koa* seedlings following two and six months of the mechanical stress treatment: (A) Visual growth differences between stressed and unstressed after two months of treatment and (B) six months of treatment; (C) quantification of the stem thickness of stressed and unstressed; (D) quantification of the stem heights of stressed and unstressed; (E) quantification of the maximum leaflet length of stressed and unstressed; (F) quantification of the root dry weight of stressed and unstressed. The stressed and unstressed plants are labeled as S and U, respectively. In (C-F), n = 39 for each of the 2-month-old stressed and unstressed groups, and n = 15 for each of the 6-month-old stressed and unstressed groups. An asterisk (*) indicate significant changes in the stressed compared to the unstressed control by two-tail t-test ($p < 0.05$), and error bars represent standard errors.

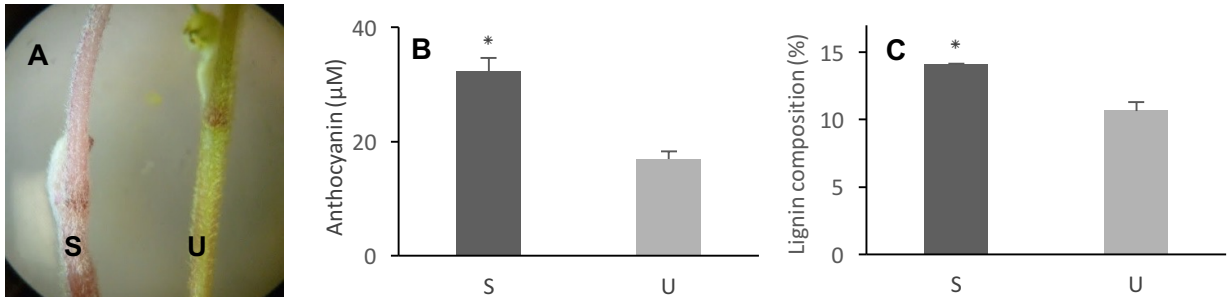

Figure 19. Quantifications of anthocyanin and lignin from stems of *Acacia koa* seedlings following two months of mechanical stress treatment: (A) Visual stem anthocyanins in stressed and unstressed; (B) quantification of anthocyanin concentration in stems of stressed and unstressed; (C) quantification of lignin composition in hypocotyls of stressed and unstressed. The stressed and unstressed plants are labeled as S and U, respectively. In (B), n = 14 for each of the stressed and unstressed group; in (C), n = 3 for each of the stressed and unstressed group, where n represents a pool of 10 hypocotyls. An asterisk (*) indicate significant changes in the stressed compared to the unstressed plants by two-tail t-test (p < 0.05), and error bars represent standard errors.

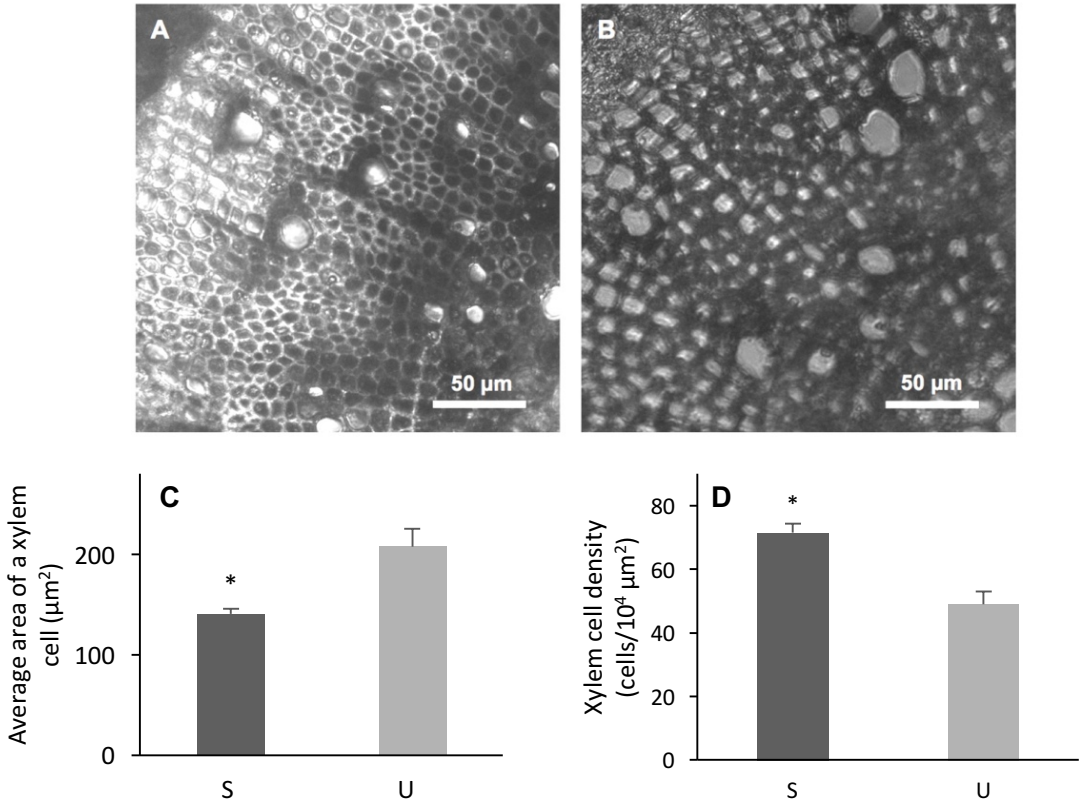

Figure 20. Microscopic analysis of cross sections of xylem in two-month-old stressed and unstressed *Acacia koa* seedlings: (A) stem cross section of the stressed; (B) stem cross section of the unstressed; (C) quantification of average xylem cell size in the stressed and unstressed; (D) quantification of xylem cell density in the stressed and unstressed *A. koa* seedlings. The stressed and unstressed plants are labeled as S and U, respectively. In (C) and (D), n = 3 for each of the stressed and unstressed group. An asterisk (*) indicate significant changes in the stressed compared to unstressed by two-tail t-test (p < 0.05), and error bars represent standard errors.

5.3.4. Genes induced by mechanical stress

The microarray analysis indicated that out of the 4,000 cDNA sequences, 60 sequences were upregulated more than two-fold in the mechanically stressed plants within 60 min following the treatment (Fig. 21A). Those 60 sequences were categorized according to their putative functions; 28 were classified in transcriptional regulation, 14 in signal transduction, 5 in carbohydrate metabolism, 4 in protein degradation, 3 in disease resistance, and 2 in hormone metabolism (Fig. 21B). Some of the most upregulated genes are those that have homology with RING finger protein (> 18-fold), zinc-finger transcription factor (> 15-fold), ethylene-responsive-element binding proteins (EREBPs; > 9-fold), WRKY transcription factor (> 8-fold), protein phosphatase 2C (> 8-fold), dehydration responsive element binding (DREB) protein, and disease resistance protein (> 4-fold; Table S4).

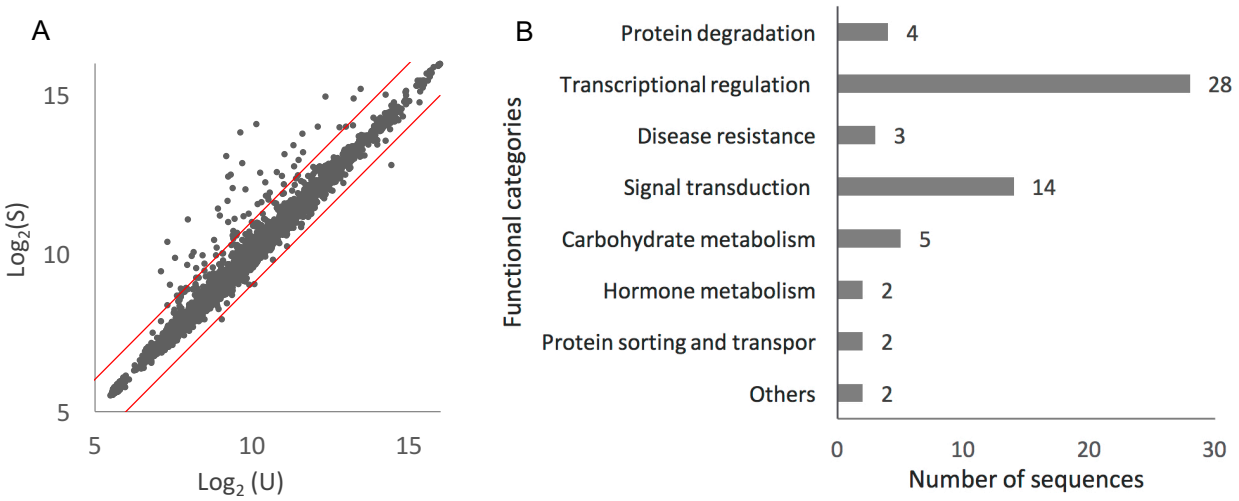

Figure 21. Microarray analysis of 4,000 genes that may be related to plant defense and growth in *Acacia koa*. (A) A scatter plot of $\log_2$ of the signal intensities comparing the gene expressions in stressed (S) and unstressed (U). Each group had two biological replicates (n = 2). In stressed, n represents pools of stems from 30 plants collected at 10, 30, and 60 min after the treatment (10 plants for each time point); in unstressed, n represents pools of 20 unstressed stems. The two lines represent a cutoff for > 2-fold change in expression; those genes induced more than 2-fold were analyzed in detail. (B) Functional categorization of genes determined to be upregulated by mechanical stress through the microarray analysis. Sixty sequences shown to be upregulated by mechanical stress more than two-fold were categorized into functional groups according to their closest annotations obtained from BLASTX.

The NanoString nCounter analysis verified upregulation of 53 sequences out of the 60, which was further confirmed by qRT-PCR (Table 8; Fig. 22). The expression levels of most of the sequences were low (~2-fold) at 10 min following the stress treatment but peaked at 30 min. Two sequences that have homology with a RING finger protein (TSA accession number

GBYE01027655) or a zinc finger protein (ZFP; TSA accession number GBYE01019260, respectively) had the highest fold-change of ~11 at 10 min, showing a rapid response of the plant to the mechanical stimulus. The sequence of the putative RING finger protein had the highest expression of 38.1-fold at 30 min after the treatment, whereas the sequence of the putative ZFP was upregulated 45-fold within 30 min. The most represented putative function among the 53 upregulated sequences was ethylene-related transcription factors (9 sequences homologous to AP2/ERF, DREB, and EREBPs), followed by WRKY transcription factors (6 sequences), and calcium-binding proteins (5 sequences). Three sequences homologous to disease-resistance proteins were also confirmed to be induced by mechanical stress, and they were upregulated more than 10 times at 30 min following the stress treatment.

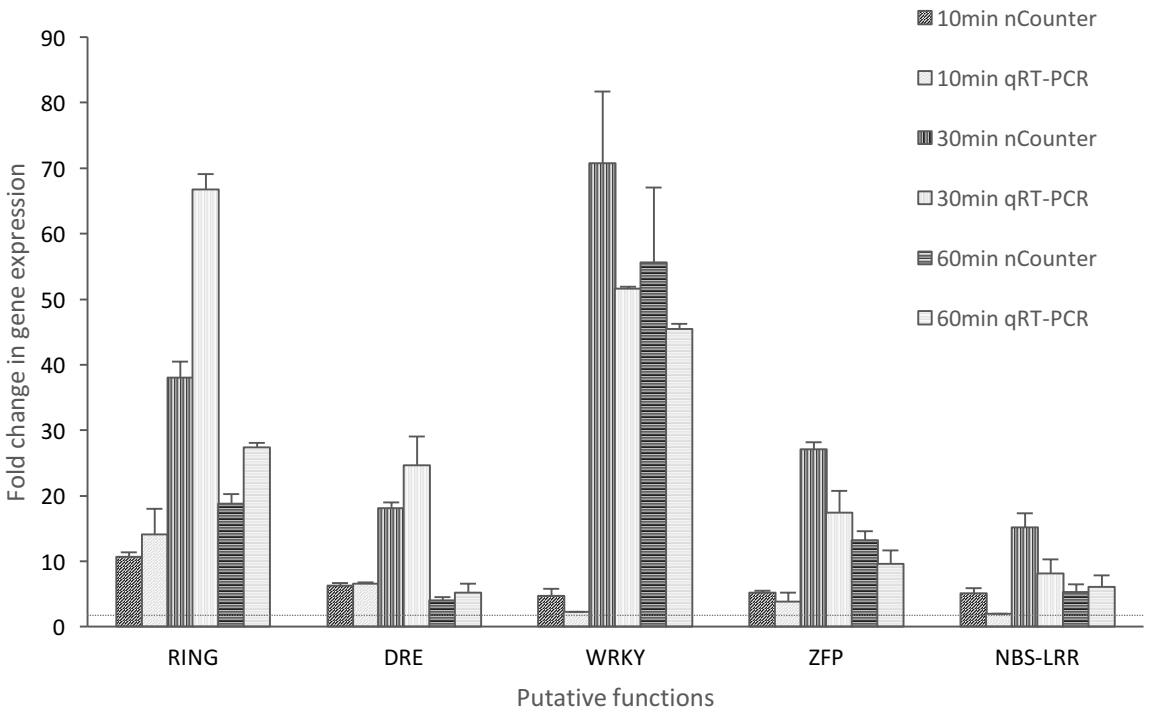

Figure 22. Verification of expression levels obtained from the NanoString nCounter analysis by quantitative real-time PCR (qRT-PCR). The gene expression levels were verified in five sequences at three different time points 10, 30, and 60 min following the mechanical stress treatment relative to unstressed control. The data shows a correlation between the data obtained from the two methods. Abbreviations: RING, RING finger protein; DRE, dehydration responsive element-binding protein; WRKY, WRKY transcription factor; ZFP, zinc finger protein; NBS-LRR, nucleotide-binding site leucine-rich repeat disease resistance protein.

Table 8. Fold change of the genes at 10, 30, and 60 min following stress treatment, determined by comparing expression levels of stressed to unstressed *Acacia koa* using NanoString nCounter analysis

Acc. No.	Putative function	Expression fold change					
		10 min		30 min		60 min	
		Ratio	SE	Ratio	SE	Ratio	SE
Protein degradation							
GBYE01027655	RING finger protein, ALT family	10.68	0.72	38.06	2.38	18.81	1.47
GBYE01011012	RING finger protein	5.93	0.33	15.73	0.65	7.20	0.46
GBYE01014767	RING finger protein, ALT family	1.87	0.18	3.41	0.22	3.41	0.16
GBYE01007181	E3 ubiquitin ligase	1.81	0.15	3.50	0.11	4.48	0.15
Transcriptional regulation							
GBYE01007979	AP2 domain class transcription factor	2.45	0.33	3.66	0.30	3.58	0.29
GBYE01025728	AP2 domain-containing transcription	2.90	0.14	18.74	1.93	31.43	4.86
GBYE01041899	AP2/ERF domain-containing protein	2.49	0.34	6.24	0.92	3.57	0.54
GBYE01036775	AP2/ERF domain-containing protein	2.69	0.23	6.66	0.57	3.54	0.33
GBYE01014032	Auxin-induced protein	(1.36)	0.29	4.74	0.69	3.81	0.48
GBYE01048118	DRE binding protein	6.33	0.33	18.15	0.88	4.06	0.45
GBYE01071204	DRE binding protein	7.95	1.40	56.91	6.13	66.68	11.17
GBYE01008127	Ethylene-responsive element-binding protein	3.71	0.24	8.63	0.40	6.08	0.60
GBYE01017658	Ethylene-responsive transcription factor	4.61	0.33	26.58	2.11	21.16	2.05
GBYE01005208	Ethylene-responsive transcription factor	2.12	0.24	5.65	0.27	8.92	0.55
GBYE01026684	MYB transcription factor	1.40	0.04	6.09	0.59	11.36	1.06
GBYE01030298	MYB transcription factor	1.47	0.08	5.55	0.47	9.98	0.80
GBYE01024450	NAC domain protein	1.68	0.18	10.27	1.10	26.38	3.46
GBYE01010007	NAC domain protein	2.44	0.49	6.88	0.46	7.91	0.72
GBYE01039799	WRKY transcription factor	5.25	0.76	58.71	5.25	37.01	5.82
GBYE01016099	WRKY transcription factor	2.13	0.25	15.47	1.03	14.31	1.81
GBYE01050872	WRKY transcription factor	2.19	0.40	11.64	1.10	13.39	1.24
GBYE01005028	WRKY transcription factor	1.68	0.15	3.68	0.21	3.51	0.30
GBYE01041303	WRKY transcription factor	4.72	1.04	70.75	10.92	55.66	11.37
GBYE01004300	WRKY transcription factor	2.15	0.26	9.32	0.82	14.86	1.62
GBYE01019260	Zinc finger protein	11.42	0.87	45.00	2.23	31.36	2.93
GBYE01019654	Zinc finger protein	5.21	0.30	27.14	1.02	13.22	1.34
GBYE01022591	Zinc finger protein	2.93	0.21	7.41	0.48	7.99	0.71
Disease resistance							
GBYE01072738	CC-NBS-IRR resistance protein	8.72	2.17	19.15	2.31	10.29	1.60
GBYE01078745	NBS-LRR disease-resistance protein	5.08	0.80	15.17	2.13	5.32	1.19
GBYE01050160	NBS-LRR disease-resistance protein	3.93	0.15	10.41	0.56	6.09	0.47
GBYE01006613	Respiratory burst oxidase homolog	1.11	0.05	1.40	0.04	2.23	0.10

Note: n = 3 for each treatment, where *n* represents 10 *A. koa* stems; $p < 0.05$ unless in prentices; $p \geq 0.05$ for those in prentices.

Table 8. (cont.) Fold change of the genes at 10, 30, and 60 min following stress treatment, comparing stressed to unstressed determined using NanoString nCounter analysis

Accession No.	Putative function	Expression fold change					
		10 min		30 min		60 min	
		Ratio	SE	Ratio	SE	Ratio	SE
Signal transduction							
GBYE01006842	Calcium-binding EF-hand protein	1.99	0.03	7.25	0.28	8.12	0.65
GBYE01006511	Calcium-binding protein	1.72	0.06	7.12	0.68	15.96	1.25
GBYE01005328	Calcium-binding protein	1.59	0.09	4.13	0.30	5.16	0.44
GBYE01000487	Calmodulin	1.23	0.05	2.47	0.08	3.67	0.19
GBYE01007399	Calmodulin binding protein	2.30	0.11	7.49	0.36	5.66	0.53
GBYE01012536	Lectin protein kinase family protein	2.13	0.26	7.22	0.64	9.31	1.13
GBYE01025941	LRR-receptor kinase	(1.48)	0.39	2.37	0.75	4.16	1.10
GBYE01007796	LysM type receptor kinase	2.53	0.22	12.02	0.97	12.29	0.77
GBYE01008850	Mitogen-activated protein kinase	5.00	0.40	24.10	1.24	23.69	2.03
GBYE01006033	Mitogen-activated protein kinase	4.31	0.31	23.17	1.05	23.20	1.94
GBYE01052680	Octicosapeptide/Phox/Bem1p (PB1)	6.60	0.45	8.67	0.34	8.56	0.98
GBYE01013735	Protein phosphatase 2C	4.89	0.28	19.97	0.74	20.03	1.57
GBYE01026109	Protein phosphatase 2C	4.71	0.35	20.52	0.70	7.30	1.18
Carbohydrate metabolism (3)							
GBYE01003680	UDP-D-glucuronic acid 4-epimerase	3.14	0.09	10.34	0.66	11.14	0.82
GBYE01007604	UDP-glucosedehydrogenase	1.40	0.06	4.87	0.25	6.25	0.72
GBYE01001472	UDP-glucuronate 5-epimerase	2.80	0.11	10.83	0.71	11.61	0.87
Hormone metabolism							
GBYE01025591	1-aminocyclopropane 1-carboxylate synthase	3.51	0.67	17.56	1.58	16.44	1.88
GBYE01014926	Abscisic acid 8'-*hydroxylase*	2.62	0.18	29.96	1.37	35.80	3.87
Protein transport and sorting							
GBYE01041529	Syntaxin	1.43	0.18	8.38	0.63	9.91	1.39
GBYE01007398	Syntaxin	1.97	0.18	5.15	0.18	5.33	0.54
Others							
GBYE01000347	Lignin biosynthetic peroxidase	(0.91)	0.15	1.69	0.29	3.38	0.57
GBYE01000925	Metal ion binding protein	2.65	0.32	6.57	0.38	5.69	0.31

Note: n = 3 for each treatment, where *n* represents 10 *A. koa* stems; $p < 0.05$ unless in prentices; $p \geq 0.05$ for those in prentices.

5.4. Discussion

Acacia koa inhabits a wide range of areas at elevations from the sea level to 2,000 m, in wet or mesic forests with annual rainfall from 1,850 to 5,000 mm (Harrington et al. 1995; Wilkinson and Elevitch 2005; Baker et al. 2009). *Acacia koa* trees show various sizes and shapes in different geological locations, and it has been reported that wood properties of *A. koa* are also influenced by environmental factors to a large extent (Loudat and Kanter 1996; Dudley and Yamasaki 2000). Mechanical stimuli, such as rain and wind, are common environmental factors that affect growth and development in plants. It is especially important to understand the effects of wind on *A. koa* because in areas with steady winds, *A. koa* plants are reported to have relatively slower growth (Elevitch et al. 2006). In this study, physical stress similar to that produced by wind was artificially created in *A. koa* seedlings through bending stems in four cardinal directions to observe morphological, physiological, and biochemical effects of such mechanical stress.

5.4.1. Physical adaptation of *A. koa* to mechanical stress

Non-wounding swaying of stems in four cardinal directions had a profound effect on the morphology of *A. koa* (Fig. 18), making it capable to withstand further mechanical stress. The reduced height and increased stem diameter after the stress treatment resembled the thigmomorphogenetic changes previously observed with other woody plants, such as *Pinus taeda* (Telewski and Jaffe 1986*a*) and *Ulmus americana* (Telewski and Pruyn 1998), and other dicotyledonous plants, including, *Solanum lycopersicum* (Coutand et al. 2000), and *Nicotiana tabacum* (Anten et al. 2005). Interestingly, little to no overall changes have been reported in some plants in which the cambium is limited or absent; for example, there were a little change in inflorescence stem diameter of *Arabidopsis* and no change in stem diameter of *Helianthus annus* following mechanical stress (Smith and Ennos 2003; Paul-Victor and Rowe 2011). Therefore, elevated cambial activity may be a major factor that increases the overall stem diameter (Biro et al. 1980; Jacobs 1953; Pruyn et al. 2000; Telewski and Jaffe 1986*a*, 1986*b*); as such, increase in xylem cells, which are derived from the cambium (Lev-Yadun and Flaishman 2001), was observed in several mechanically perturbed plants, such as *Phaseolus vulgaris* (Biro et al. 1980) and *N. tabacum* (Hepworth and Vincent 1999). Similarly, in the stressed *A. koa*, there was an increase in number of xylem cells (Fig. 20A and B); however, the proportions of the xylem and pith sizes relative to the stem size showed no significant difference between the stressed and unstressed *A. koa* (data not shown). Therefore, expansion of xylem

and also other tissues appeared to contribute to the increase in overall stem thickness. In addition, the stressed *A. koa* had denser xylems with smaller cells (Fig. 20D), and similar xylem characteristics were observed in mechanically stressed *Abies fraseri* (Telewski 1989). Therefore, thigmomorphogenetic responses in *A. koa* may include the enhancement of secondary growth and modification of wood properties; further analysis of responses to mechanical stimuli in *A. koa* may reveal unique genetic and physiological responses that will help study wood formation and development.

5.4.2. Increase in phenylpropanoid biosynthesis in stems of thigmomorphogenetic *A. koa*

The significant elevation of lignin and anthocyanin contents in the stressed *A. koa* indicated that thigmomorphogenesis affected the phenylpropanoid biosynthesis pathway, which is responsible for synthesis of a wide variety of secondary metabolites, including monolignols and flavonoids (Mayer et al. 2001; Fig. 19). Monolignols are monomers of lignin, an important component of wood that constitutes 25-35% of the secondary cell wall (Plomion et al. 2001); the stressed *A. koa* should have higher deposit of lignin and therefore may have thicker cell walls and stronger structural support (for review, see Vanholme et al. 2010) so that they would have higher resistance against the swaying movement. In *Bryonia dioica* and *S. lycopersicum*, increased lignin and elevated activities of enzymes involved in lignification, such as phenylalanine ammonia-lyase (PAL) and cinnamyl alcohol dehydrogenase (CAD), have been observed 6 h following the mechanical stimulus (Saidi et al. 2009; de Jaegher et al. 1985). However, the genes for enzymes involved in the monolignol biosynthesis, including PAL and CAD, were not induced in *A. koa* within 60 min following the mechanical stress. Since the expression levels of the genes beyond 60 min were not determined in this study, further studies will be needed to elucidate effects of mechanical stress treatment on the transcription of the phenylpropanoid pathway genes in *A. koa*.

The stressed *A. koa* had enhanced concentration of anthocyanins, a type of flavonoids; such increases in anthocyanin levels due to the mechanical stress have not been reported in other studies of thigmomorphogenesis. On the contrary, decrease in anthocyanin content has been observed in thigmomorphogenetic *C. papaya* (Porter et al. 2009). It shows that molecular responses to mechanical stress vary among species as anthocyanin production was repressed in the stressed *C. papaya*, while it was enhanced in the stressed *A. koa.* Anthocyanins and other flavonoids are derived from *p*-coumaroyl CoA, a major pivotal intermediate of the phenylpropanoid pathway, which is also an intermediate product for biosynthesis of monolignols

and other phenylpropanoids. It is the substrate for the monolignol biosynthesis enzymes, including hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) and caffeoyl-CoA *O*-methyltransferase (CCoAOMT), as well as for the flavonoid biosynthesis enzyme chalcone synthase (CHS). Therefore, it is likely that *p*-coumaroyl CoA accumulated in response to the mechanical stress, feeding both pathways of monolignol and flavonoid biosynthesis. Lignin production is shown to be induced by mechanical perturbations in several other plants (de Jaegher et al. 1985; Bourgeade et al. 1989; Porter et al. 2009) while anthocyanin production is not; therefore, the anthocyanins in the stressed *A. koa* may be inadvertently produced due to enhancement of the lignin biosynthesis pathway.

It is also possible that thigmomorphogenetic *A. koa* produced other phenylpropanoids, such as coumarins, tannins, and other aromatic compounds, from *p*-coumaroyl CoA (VanEtten et al. 1989; Vogt 2010). Many of these compounds, including lignin, are known to be involved in plant defense mechanisms in various plants (Uppalapati et al. 2009; Kamphuis 2012; Purwar et al. 2012; Boubakri et al. 2013). Therefore, besides being more tolerant to mechanical stress, the thigmomorphogenetic *A. koa* plants may also grow more tolerant to other stresses, including resistance to pathogens, compared to the unstressed *A. koa* plants; further analysis of the mechanically stressed plants by exposing them to pathogens will verify if mechanical stress induces disease resistance in plants.

Regulation of chlorophyll biosynthesis in leaves or total tissues in thigmomorphogenetic plants has been reported. For instance, chlorophyll content went up in *Medicago truncatula*, *Apium graveolens*, *Lactuca sativa*, and *S. lycopersicum* (Biddington and Dearman 1985; Mitchell et al. 1975; Tretner et al. 2008) whereas it decreased in *B. oleracea* and *C. papaya* (Biddington and Dearman 1985; Porter et al. 2009). On the contrary, in the present study, chlorophyll content in the stem, including petioles, did not show any significant difference between the mechanically stressed and unstressed *A. koa*. Chlorophyll content of the leaves was not determined in this study.

5.4.3. Calcium signaling in thigmomorphogenesis of *A. koa*

Calcium (Ca^{2+}) is one of the major signaling molecules released from the endoplasmic reticulum to the cytosol in response to mechanical perturbations (Braam 2005; Telewski 2006; Chehab et al. 2009). Studies have shown upregulation of genes for calmodulins and calmodulin-related proteins in various plants, including *Arabidopsis, C. papaya, Brassica napus*, and *Vigna radiata* (Braam and Davis 1990; Botella and Arteca 1994; Botella et al. 1996; Oh et al. 1996;

Lee et al. 2005; Porter et al. 2009). The gene for calmodulin was one of the first identified touch-inducible (TCH) genes (TCH1; GenBank accession number AAA32762.1; Braam and Davis 1990) in *Arabidopsis*, and its homolog in *A. koa* (TSA accession number GBYE01000487; 97.6% sequence identity) was among the five sequences coding for putative calcium-binding proteins that are upregulated in response to mechanical stress (Table 8). Moreover, in stressed *A. koa*, two putative mitogen-activated protein kinase (MAPK) sequences were upregulated more than 20-fold within 30 min following the stress treatment. MAPK sequences are known to be indirectly or directly regulated by Ca^{2+} in plants (Wurzinger et al. 2011). The increased expression levels of genes encoding MAPK and other protein kinases, possibly calcium-dependent, following mechanical stimulation have been observed in other plants (Seo et al. 1995; Mizoguchi et al. 1996; Bögre et al. 1997). Therefore, it is possible that calcium signaling may induce MAPK cascades in the stressed *A. koa* as well.

5.4.5. Regulation of phytohormones in thigmomorphogenesis in mechanically stressed *A. koa*

Ethylene (ET), a plant growth hormone, appears to be involved in early thigmomorphogenetic responses in *A. koa*. A sequence for putative 1-aminocyclopropane 1-carboxylate synthase (ACS), a key enzyme for ET production, was rapidly induced within 10 min following the stress (Table 8). In addition, the expression of nice sequences for putative ET-responsive transcription factors was also enhanced. In other plants, ET production is shown to be one of the early thigmomorphogenetic responses (de Jaegher et al. 1987; Botella et al. 1995; Arteca and Arteca 1999; Tatsuki and Mori 1999). Increase in the activity of ACS and upregulation of an ET-responsive transcription factor were also observed rapidly following mechanical stimulation in *Phaseolus vulgaris* and *Arabidopsis* (Biro and Jaffe 1984; Lee et al. 2005). Subsequent to exogenous application of ET, stunted growth was observed in various plants, resembling thigmomorphogenetic responses (Jaffe and Biro 1979; de Jaegher et al. 1987; Erner and Jaffe 1982). However, ET-insensitive *Arabidopsis* mutants still showed thigmomorphogenetic changes following mechanical stimulation, so ET is unlikely to be the 'primary' signaling molecule (Johnson et al. 1998), at least in *Arabidopsis.*

Another growth phytohormone, abscisic acid (ABA) may be involved in thigmomorphogenesis in *A. koa*. Many plants, such as *H. annuus* and *Zea mays*, had increased ABA levels following the mechanical stimulation (Whitehead and Luti 1962; Beyl and Mitchell 1983), and exogenous application of ABA also led to morphological changes similar to

thigmomorphogenetic responses (Erner and Jaffe 1982). On the contrary, a sequence for a key enzyme for ABA degradation, ABA 8'-hydroxylase, was upregulated in the stressed *A. koa* (> 35.8-fold within 60 min following the stress); thus, the induction of the gene would result in reduction of ABA levels in thigmomorphogenetic *A. koa*. It may be possible that ABA synthesis was downregulated due to feedback inhibition. Different plants respond to mechanical stress differently; further analyses on phytohormones and their quantification in the stressed and unstressed *A. koa* may elucidate their roles in thigmomorphogenesis.

5.4.6. Abiotic- and biotic-stress-related genes induced by mechanical stress in *A. koa*

The gene expression analyses suggested that thigmomorphogenetic responses in *A. koa* might influence a wide range of cellular mechanisms, such as transcriptional regulation, signal transduction, carbohydrate metabolism, and hormone metabolism. Interestingly, many of them are related to abiotic and biotic stresses as well as plant growth and development. Among the upregulated 23 sequences coding for transcription factors, 15 sequences encode putative WRKYs, MYBs, NACs, DREBs, and ZFP, many of which are known to be involved in disease resistance, defense, and abiotic stress tolerance, such as drought and high-salinity stresses (Lata and Prasad 2011; Nuruzzaman et al. 2013; Ambawat et al. 2013; Bakshi and Oelmüller 2014). Among the transcription factors, two sequences for ZFP were highly upregulated within 10 min after the treatment, and they were found to be homologous to a ZAT-10 protein that is expressed in response to drought, cold, salt, and photo-oxidative stresses in *Arabidopsis* (Mittler et al. 2006; Rossel et al. 2007). Among the 57 tested sequences in the nCounter analysis, it is one of the most rapidly upregulated genes in response to the mechanical stress. The upregulation of these abiotic stress-related transcription factors suggests that the mechanically stressed *A. koa* may also develop higher tolerance to other environmental stresses.

It is notable that sequences for disease resistance (R) proteins, or nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, were induced by mechanical perturbations in *A. koa*. R proteins are involved in disease resistance as they recognize invading pathogens by directly binding to or indirectly monitoring effector molecules secreted by pathogens (McHale et al. 2006). Although the mechanically stressed *A. koa* were not exposed to pathogens, three sequences encoding R proteins were upregulated over 10-fold within 30 min after the stress treatment (Table 8). Similarly, the microarray analysis of the mechanically inducible genes in *Arabidopsis* showed upregulation of 14 R proteins (> 2-fold; Lee et al. 2005). In addition to R proteins, ATL family RING finger ubiquitin ligase is shown to regulate defense mechanisms and

cell death regulations in response to pathogenic toxins (Guzmán 2012). One homolog of ATL family RING finger ubiquitin ligases was highly upregulated in response to the mechanical stress in *A. koa*, increasing by over 10-fold within 10 min and over 38-fold within 30 min following the mechanical stress (Table 8). Higher expression levels of the R proteins and ATL family RING finger proteins in the stressed *A. koa* indicates that mechanical stimulation may also induce tolerance to pathogens.

5.4.7. Conclusion

In this study, morphological, biochemical changes and levels of gene transcription due to mechanical stress were determined in *A. koa*. Prolonged mechanical stimulation led to stunted stem length and thickened stem diameter with a greater number of xylem cells. Anthocyanin and lignin were accumulated in the stems of mechanically stressed *A. koa*, indicating that mechanical stress induces the phenylpropanoid biosynthesis pathway. The gene expression analyses showed upregulation of genes in a wide range of cellular mechanisms in response to mechanical stress, suggesting complex signaling pathways of thigmomorphogenesis. Genes for calcium signaling, such as calmodulin and *MAPK*, ET, and ABA metabolisms, and stress-related transcriptional regulation were highly expressed. Two of the most rapidly upregulated genes encode RING finger protein and ZFC, and they may be related to abiotic and biotic stresses, such as drought and disease resistance. Surprisingly and interestingly, genes for disease resistance, which are generally induced as a result of biotic stress, were also induced by mechanical stress alone. This result suggests that trees may develop disease resistance in response to mechanical stress applied by strong wind and heavy rain in natural forests. It also suggests that a simple mechanical stress may be used to identify genetic as well as biochemical factors that influence disease resistance; these molecules may be useful as potential markers for seedling selection in *A. koa* improvement programs.

CHAPTER 6
RESULTS FOR SPECIFIC OBJECTIVE 3

Mechanical stress induces resistance against infection by *Fusarium oxysporum* in *Acacia koa* through enhancement of drought tolerance, antifungal compounds, and expression of genes for defense enzymes

6.1. *Introduction*

Acacia koa (koa) is a leguminous timber tree endemic to the Hawaiian Islands. Its timber is a highly priced commodity due to the beautiful texture, hardiness, and the carving quality of the wood. With a high market value, priced at up to $125 per board foot, *A. koa* produces the gross value in the range of $20-$30 million annually, comprising a majority of Hawai'i's forestry (Yanagida et al. 2004; Baker et al. 2009). As such, *A. koa* serves as a vital economical resource for the Hawaiian Islands. However, *A. koa* forests have been suffering from a devastating wilt disease caused by the fungal pathogen *Fusarium oxysporum* f. sp. *koae* (Pejchar and Press 2006). Besides wilting, the fungus causes crown and shoot dieback, and once trees are infected, mortality rates are high (Anderson et al. 2002; Baker et al. 2009; Elevitch et al. 2006). Because the fungus can survive an extended period of time in the soil without its hosts, it is difficult to control this ubiquitous and hardy pathogen. To protect koa forestry from the disease, it is crucial to understand how disease resistance is conferred in koa.

Our previous study of thigmomorphogenesis in *A. koa* showed that non-wounding mechanical stress induced expression of several genes involved in disease resistance. The mechanical stress also elevated the accumulation of phenolic compounds, such as lignin and anthocyanins, which are often induced in defense response. Based on these results, it was hypothesized that mechanically stressed *A. koa* plants have increased disease resistance against infection by *F. oxysporum.* Therefore, studying mechanically stressed *A. koa* may be used as a model to study disease resistance, and it can be used to elucidate defense mechanisms and to identify key molecules that influence disease resistance.

The goal of this study was to understand the mechanisms underlying how mechanical stimulation can induce disease resistance at the molecular and biochemical levels in *A. koa*. Here, the focus was on biochemical compounds that are involved in shikimate and phenylpropanoid biosynthesis pathways that leads to production of a wide variety of secondary metabolites. The gene for phenylpropanoid biosynthesis enzymes have been also shown to be up-regulated in response to infection by pathogens (Conrath 2006). Phenylpropanoid biosynthesis enzymes produce a wide variety of secondary metabolites,

many of which have antioxidant and antimicrobial activities. The initial molecule of the phenylpropanoid biosynthesis pathway, phenylalanine, is produced in the shikimate biosynthesis pathway, so the genes involved in this pathway may be also important in plant defense. The study also focused on genes for pathogenesis-related (PR) proteins, which are plant enzymes that accumulate in pathological conditions. There exists a wide variety of PR proteins, especially, chitinases and β-1,3-glucanases (Bgl), which are considered to be important enzymes for protection against fungal infections since they have been observed to be induced by exposure to fungal pathogens (Broekaert et al. 2000). These enzymes can break down primary components of fungal cell walls, including chitin and β-1,3-glucan, especially in the tips of hyphae and germ tubes (van Loon and van Strien 1999; Broekaert et al. 2000), so they may be able to inhibit fungal growth. Rushanaedy et al. (2012) has identified the genes for four chitinases that were up-regulated in response to *F. oxysporum* infection in *A. koa*.

In the present study, 15 genes involved in shikimate and phenylpropanoid biosynthesis pathways and one gene for Bgl were identified. The study determined if mechanical stimulus elevates the expression levels of those genes and two previously isolated chitinase genes. It also determined the level of resistance in the mechanically stressed *A. koa* seedlings against infection by *F. oxysporum,* the expression levels of the identified genes in response to *F. oxysporum* infection, and antifungal activities in mechanically stressed *A. koa* seedlings.

6.2. *Materials and Methods*

6.2.1. Isolation and sequencing of target cDNA

The cDNA sequences for shikimate and phenylpropanoid biosynthesis enzymes and pathogenesis-related (PR) protein were searched and isolated from the transcriptomes sequences of *A. koa,* previously annotated with protein functions by Ishihara et al. (2015). To determine if the transcriptome sequences include complete coding sequences (CDSs), all possible open reading frames (ORFs) were predicted using NCBI Open Reading Frame (ORF) Finder. The longest predicted ORFs were compared with the known sequences of the target enzymes in other plants through BLASTX analysis to assess if they were correct. Similarly, the genes for the RING and zinc finger proteins, the most expressed sequences induced immediately following the mechanical stress treatment in the previous chapter, were also determined.

Following the identification of CDSs, a pair of primers targeting the 3' and 5' untranslated regions (UTRs) was designed for reverse transcription PCR (RT-PCR) analysis for each gene to amplify the CDS (Table S5). Briefly, the improved RNA extraction method and cDNA synthesis with an oligo(dT) primer were performed as described by (Ishihara et al. 2016). The RT-PCR analysis was run in a 20 µL PCR reaction consisting of 0.25 µL forward primer (10 µM), 0.25 µL reverse primer (10 µM), 10 µL GoTaq ® Colorless Master Mix (Promega), and 1 µL of the synthesized cDNA. Reaction conditions were 95°C for 5 min, 35 cycles of 94°C for 30 s, 54°C for 90 s, 72°C for 30 s, with a final annealing step at 72°C for 5 min.

The amplified CDS for each gene was sequenced through Sanger sequencing at Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB), University of Hawai'i at Mānoa, Honolulu, HI. The nucleotide sequences were submitted to GenBank with accession numbers KX784933-KX789451. The present study also included two other PR genes previously identified by Rushanaedy et al. (2012), class I chitinase (*Akchit1a*, GenBank accession number JQ677143*)* and class III chitinase (*Akchit3,* GenBank accession number JQ677146).

6.2.2. Plant growth and treatment

Seeds of *A. koa* were collected from the Maunawili Research Station of Hawai'i Agriculture Research Center (HARC), Kailua, Hawai'i. Seeds were immersed in concentrated sulfuric acid for 10 min for scarification, rinsed with sterilized water six times, and incubated in petri dishes with wet paper towels at 28 ^{o}C till they germinated in 3-5 days (Rushanaedy et al. 2012). The resulting germinated seedlings were then planted into 4-inch pots containing a vermiculite-perlite mixture pre-wetted with Hoagland's solution. The seedlings were grown at 25 °C $\pm$ 2 °C with a 16/8-h light/dark photoperiod with an irradiance of 30 µmol s^{-1} m^{-1} for a month. Plants were watered with Hoagland's solution once in two days. Following a week after seedlings were transferred to pots, stems were gently bent from its position at 90º with the ground level to a position at approximately 60º with the ground level in each of all the four cardinal directions manually. The seedlings in the control group were maintained similarly, except that they were not given any mechanical stress.

6.2.3. Quantitative real-time PCR (qRT-PCR) and NanoString nCounter analyses of the mechanically stressed *A. koa*

Stems were harvested after 2, 4, 6, 12, 24, and 48 h following the non-wounding mechanical stress treatment, and they were flash-frozen in liquid nitrogen after the removal of leaves. To extract total RNA, the frozen stems were ground to a fine powder in liquid nitrogen with a mortar and a pestle that were previously baked for 6 h at 300 °C prior to use. Total RNA was extracted with the modified method using Qiagen RNeasy® Plant Kit (Valencia, CA, USA) and Fruit-mate (Takara, Japan) as described by Ishihara et al. (2016). RNA was treated with TURBO DNA-free Kit (Ambion, CA, USA) to remove genomic DNA contamination. The quantity and quality of the RNA were assessed at wavelengths of 230, 260, and 280 nm using a NanoDrop ND-1000 (NanoDrop Technologies, DE, USA).

Quantitative real-time PCR (qRT-PCR) was performed to analyze differential gene expression of *A. koa* at 2, 4, and 6 h following the mechanical treatment. First-strand cDNA was synthesized from 2 µg of DNase-treated RNA using M-MLV Reverse Transcriptase (Promega, WI, USA) with random hexamers according to the manufacturer's instructions. The qRT-PCR analysis was performed using a 10 µL PCR reaction consisting of 0.25 µL forward primer (10 µM), 0.25 µL reverse primer (10 µM), 5 µL PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Foster City, CA), and 0.5 µL of 1:10 diluted first strand cDNA. Reaction conditions were 50 °C for 2 min, 95 °C for 3 min, 40 cycles of 95 °C for 15 s, annealing for 30 s, and 72 °C for 30 s, followed by melting curve analysis of the amplicon to confirm the specificities of primers. Appropriate annealing temperature was used for each set primers (Table S6). The reaction was performed using StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). Each assay consisted of three technical replicates for three biological replicates, each consisting of 10 individual plants. The PCR protocol produced a PCR efficiency of 90% to 110% for each primer set.

To select internal reference genes for relative quantification of target gene expressions, five reference candidate genes: *ef1α*, β-actin, 18S rRNA, 5.8S rRNA, and ubiquitin-5 were tested on first strand cDNA samples from the *A. koa* stem with the primer sequences as described in Negi et al. (2011). The qRT-PCR protocol was performed as described previously, with the annealing temperature at 58 °C. The cycle threshold (Ct) values of the candidate genes were used to evaluate their expression stability by using NormFinder applet for MS Excel (Andersen et al. 2004). NormFinder allows the user to determine the stability value for each candidate gene and combinations of candidate genes. A gene or a combination of two genes with the lowest stability value were selected as a

reference control to normalize the expression levels at each time point. The gene 5.8S rRNA had the lowest stability value, so it was selected as the reference control (Table 9). The fold changes of gene expression levels relative to the unstressed control plants were determined using the selected reference genes for normalization and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Statistical significance for increase in gene expression levels in the stressed plants compared to unstressed plants was determined using Student's one-tailed t-test with a cutoff *p*-value of 0.05.

Table 9. Stability values of candidate reference genes for quantitative real-time PCR (qRT-PCR) analysis at 6 h following mechanical stress

Gene names	6 h
ef1α	0.274
18S rRNA	0.259
ubiquitin-5	0.473
5.8S rRNA	0.079
β-actin	0.182
The best combination (5.8S rRNA and β-actin)	0.100

To analyze differential gene expression of *A. koa* at 6, 12, 24, and 48 h following the mechanical treatment, a digital multiplexed gene-expression analysis called the NanoString nCounter System was used at NanoString Technologies, Seattle WA. Briefly, total RNA extracted (100 ng) from mechanically stressed and unstressed seedlings at each time point was directly hybridized with gene-specific color-coded probes. Data collection was carried out in the nCounter Digital Analyzer as described by the manufacturer (NanoString Technologies). The NanoString Codeset was designed and synthesized by NanoString Technologies; it included the sequences for the phenylpropanoid biosynthesis enzymes isolated in a previous study (Ishihara et al. 2015) as well as reference genes of β-actin and ubiquitin-5, six positive-control, and eight negative-control probes to normalize the results. Three biological replicates, each consisting of 10 individual plants, were tested for each time point. The data analysis was performed through nSolver Analysis Software (NanoString Technologies) using the default settings. Statistical significance for increase in gene expression levels in the stressed plants compared to unstressed plants was determined using Student's one-tailed t-test with a cutoff *p*-value of 0.05.

6.2.4. Extraction of metabolites and quantification of total phenolic content

The stems of *A. koa* seedlings that are mechanically treated daily for three weeks were used for the analysis of secondary metabolites along with the unstressed control plants. Each sample consisted of 10 individual *A. koa* seedlings. The samples were ground to a fine powder in liquid nitrogen; metabolites were extracted from 100 mg by sonicating with 300 µL of 90 % aq. methanol with 0.125% formic acid for 15 min. Assuming a water content of about 80% in *A. koa* stems, this would result in a final concentration of ~70% methanol and ~0.1% formic acid. Following the sonication, samples were centrifuged at 15,000 rpm for 15 min to remove insoluble debris. These extraction steps were repeated two more times, and the extracted supernatants were pooled together and stored in -20 ºC until ready to use for phenolic content analyses.

To quantify total phenolic contents in the extract from *A. koa* stems, the Folin-Ciocalteu (FC) micro-method was used according to Cicco et al. (2009). For each sample, 50 µL of the methanolic extract was diluted 1:2 with 50 µL of water in an Eppendorf tube, and 100 µL of FC reagent was added and mixed well. Exactly at 2 min after mixing, 800 µL of a 5% w/v sodium carbonate solution was added and mixed well. The mixture was incubated at 40 ºC for 20 min and then immediately cooled on ice. All the measurements were done in triplicate and the absorbance was read at 745 nm with a UV-visible spectrophotometer, UV-1600PC (VWR, PA, USA). Ten different concentrations of gallic acid were analyzed in triplicates as a reference standard. The results were expressed as mg gallic acid equivalents (GAE) per gram of the stem tissue. Also, stem lignin analysis was conducted by the Agricultural Diagnostic Services Center, University of Hawai'i at Mānoa as described in the previous chapter.

6.2.5. Reverse-phase high-performance liquid chromatography (RP-HPLC) and tandem mass spectrometry (MS/MS) analyses

To determine secondary metabolites induced by the mechanical stress, reverse-phase high-performance liquid chromatography (RP-HPLC) analysis was performed. Two hundred microliters of the methanolic extracts obtained as described above was diluted 1:5 with 800 µL of 2 % v/v aq. acetic acid. The diluted samples were centrifuged twice at 15,000 rpm for 20 min, and 500 µL aliquots were for stored in -20 ºC until ready to use for the analysis. RP-HPLC analysis was performed with a Waters 2695 automated HPLC system (Milford, MA) interfaced with a Waters 996 Photodiode Array Detection (PDA), scanning at 210–600 nm. The solvents used are: Solvent A, 2 % v/v aq. acetic acid; Solvent B,

acetonitrile. The gradient was 0-5 min, 5% B; 5-30 min, 5-35% B; 30-32 min, 35-80% B; 32-37 min, 80% B. A 100-µL volume of the samples was injected into onto a Kinetex C18 column (Phenomenex; 2.6 µm; 100A; 100 x 4.6 mm) at a flow rate of 0.7 mL/min with a 3.5-min re-equilibration period between sample injections. Using Waters Empower Pro software, resulting chromatographic profiles were extracted at 280 nm to identify any peaks that had higher amounts in the stressed *A. koa* compared to the unstressed control. Relative quantity of the compound was calculated by comparing the average areas under the peak obtained from the mechanically stressed and unstressed *A. koa* seedlings. The fractions of peak at 18 min, which was higher in the stressed plats than in the unstressed control, was suspected to be malonyl tryptophan. For a reference standard, five different concentrations (2.5, 5, 7.5, 10, and 12.5 µg/mL) of malonyl-tryptophan (product no. 80494, PhytoLab, Germany) were analyzed in triplicate, allowing for the precise establishment of concentrations reported as mg per gram fresh tissue weight (mg/gfw).

For mass spectrometry analyses, the fraction of peak at 18 min was collected, dried using a SpeedVac (Vacufuge plus, Eppendorf, Hauppauge, NY), and dissolved in a carrier solvent (70/30 % v/v acetonitrile and 0.1 % v/v aq. formic acid). Mass spectrometry analyses were conducted on the collected fractions using an AB/MDS-Sciex API 3000 triple quadrupole mass spectrometer (Thornhill, Ontario, Canada), interfaced with a PE ABI 140D Solvent Delivery System. The ESI–MS system was calibrated manually in positive mode with PPG 3000 (AB/MDS-Sciex) to achieve < 5-ppm mass accuracy, as per manufacturer's protocol. Samples were delivered to the atmospheric pressure ionization (API) source of the mass spectrometer using a continuous flow of 5–10 µL/min provided by a microsyringe infusion pump (Harvard Apparatus, MA, USA). Initially, full ion spectra were taken from m/z 100-600 Da to provide identification of the parent molecular mass using quadrupole-3 (Q-3). Tandem mass spectrometry (MS/MS) was performed with N_2 bombardment confined to quadrupole-2 (Q-2) with a collision cell gas thickness of 3×10^{14} atoms cm^{-2} and a collision energy (Q-0 to Q-2 rod offset voltage) typically set at ~20–40 eV. The resulting CID (daughter ion) spectra were obtained by scanning quadrupole-3 (Q-3) from m/z 100–350 Da. MS/MS data analysis was assisted with the use of Analyst Software (v1.4.1; AB/MDS-Sciex). Similarly, the MS/MS spectrum of malonyl-tryptophan was obtained to compare with the MS/MS spectra of the HPLC fractions.

6.2.6. Disk diffusion assay to assess antifungal properties of *A. koa* extracts

To isolate *Fusarium oxysporum* f. sp. *koae,* the roots of wilting *A. koa* seedlings infected with the fungus were cut into ~3 mm, surface-sterilized with 10% hydrochloric acid for approximately 10 s, and rinsed with sterilized water. They were placed on Komada agar medium, a semi-selective medium for *F. oxysporum* (Komada 1975) and inoculated for 3-4 days at 25-28 ºC. Once fungi started growing on the media, DNA was extracted according to Griffin, et al. (2002). PCR was performed to amplify a partial EF-1α gene sequence, using primers ef1 and ef2 designed by O'Donnell et al. (1998) (ef1: 5′-ATGGGTAAGGA(A/G)GACAAGAC-3'; ef2: 5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′). The products were sequenced at ASGPB, University of Hawai'i at Mānoa. The sequences were compared to the NCBI database through BLASTn and identified as *F. oxysporum*.

To evaluate the antifungal properties of the *A. koa* stem extracts, agar diffusion assay was conducted using Richards's medium (RM) with 10% sucrose (potassium nitrate 10 g, monopotassium dihydrogen phosphate 5 g, magnesium sulfate 2.5 g, ferric chloride 0.025 g, sucrose 100 g, agar 20 g). RM plates were first inoculated with fungal agar disks (~5 mm in diameter) cut from actively growing front of a 1-week-old *F. oxysporum* culture. After two days, sterile filter paper disks with a diameter of 6 mm were loaded with 50 µL of the plant extract and dried. For a negative control, 80% methanol/0.1% formic acid was loaded to a paper disk. Those paper disks containing plant extracts were placed 1.5 cm away from the fungal growth. The plates were incubated at 28 ºC for 6 days, and inhibition of the radial growth of *F. oxysporum* around the paper disks was measured. Four biological replicates and three technical replicates were run simultaneously.

6.2.7. Determination of resistance against infection by *F. oxysporum* and drought tolerance

Following three weeks of daily mechanical stress, *F. oxysporum* inoculation trials were conducted by transferring *A. koa* seedlings to sterilized, 115 ml growing containers (RLC7, Stuewe and Sons, Tangent, OR) filled with inoculation media. The inoculation media contained a mixture of ten virulent isolates of *F. oxysporum* f. sp. *koae.* HARC has previously obtained these isolates from wilt-infected koa throughout Hawai'i and determined their virulence in screening trials (Dudley et al. 2007*b*; James 2004). The inoculum was prepared according to Miles and Wilcoxson (1984) and Dudley et al. (2007*b*); it was mixed in a 1:50 (w/w) ratio, into a commercial peat moss and perlite growing media (Sunshine Mix 4, Aggregate Plus, Sungro Horticulture, Bellevue, WA). The survival rate of the seedlings was

observed over 100 days to determine disease resistance. The unstressed *A. koa* seedlings were similarly grown without mechanical stress treatment. For uninoculated controls, mechanically stressed and unstressed seedlings were grown in the same way except that the growth medium was not mixed with the *F. oxysporum* inoculum. Each 100-day inoculation trial consisted of 25-35 individual *A. koa* seedlings for each of the stressed and unstressed groups, and 3 trials were performed.

Similarly, to determine drought resistance, 10 mL of 15% PEG (MW 6,000) in Hoagland's solution was given to the plants once in 3 days for 50 days following three weeks of the daily mechanical stress, and the survival rate was determined. Each drought trial represented 15-20 individual *A. koa* seedlings for each of the stressed and unstressed groups, and 3 trials were performed.

6.2.8. Gene expression analyses following inoculation

Following 1, 2, 3, and 4 weeks of inoculation by *F. oxysporum* f. sp. *koae***,** stems were harvested via flash-freezing in liquid nitrogen. Total RNA extraction, cDNA synthesis, and qRT-PCR analysis were conducted as described earlier in the present study. The expression levels of the *A. koa* seedlings that were inoculated following the mechanical treatment were compared with the expression levels of *A. koa* seedlings that were not inoculated following the mechanical treatment. Similarly, the expression levels of the mechanically unstressed and inoculated seedlings were compared with the expression levels of the mechanically unstressed and uninoculated seedlings. Internal reference genes at each time point were selected, and relative fold change expression levels were calculated as previously described in the present study. The reference genes were selected as described previously. The lowest stability value was observed in the following gene or combination of genes for each time point, and these genes were selected as a reference control: the combination of *ef1α* and ubiquitin-5 at 1 week post-inoculation (wpi), the combination of *ef1α* and 18S rRNA at 2 wpi, the single gene of 18S rRNA at 3 wpi, and the combination of 18S rRNA and ubiquitin-5 at 4 wpi (Table 10). The fold changes of gene expression levels relative to the uninoculated control plants were determined for each of the stressed and unstressed groups using the selected reference genes for normalization and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Statistical significance for increase in gene expression levels in the stressed plants compared to unstressed plants was determined using Student's one-tailed t-test with a cutoff *p*-value of 0.05.

Table 10. Stability values of candidate reference genes for quantitative real-time PCR (qRT-PCR) analysis at 1-4 week post-inoculation (wpi)

Gene names	1 wpi	2 wpi	3 wpi	4 wpi
ef1α	0.161	0.101	0.204	0.193
18S rRNA	0.326	0.241	0.084	0.121
Ubiquitin-5	0.120	0.090	0.374	0.169
5.8S rRNA	0.464	0.457	0.224	0.284
β-actin	0.350	0.740	0.151	0.340
The best combination*	0.108	0.068	0.089	0.117

*ubiquitin-5 and ef1α for 1 wpi, ef1α and 18S rRNA for 2 wpi, 18S rRNA and β-actin for 3 wpi, 18S rRNA and ubiquitin-5 for 4 wpi

6.2.9. Detection of *F. oxysporum*

The stems of mechanically stressed and unstressed *A. koa* seedlings were collected at 1, 2, 3, and 4 weeks following the inoculation by *F. oxysporum*. Approximately 2-mm of stem pieces were cut out at 2.5 cm from the stem base of *A. koa* seedlings, and they were surface-sterilized as described previously in the present study and placed on Komada agar medium. The *A. koa* seedlings without inoculation were tested as a negative control. To verify the results, qualitative real-time PCR was performed using cDNA template from inoculated *A. koa* stems and primers specific to the internal transcribed spacer (ITS) region of *F. oxysporum* (Mishra et al. 2003), FOF1 (5'- ACATACCACTTGTTGCCTCG-3') and FOR1 (5'-CGCCAATCAATTTGAGGAACG-3'). The amplification of the ITS sequence would indicate the presence of *F. oxysporum* in the stems of *A. koa*. The RT-PCR reaction (10 µL/reaction) consisted of 0.25 µL forward primer (10 µM), 0.25 µL reverse primer (10 µM), 5 µL PowerUp™ SYBR® Green Master Mix (Applied Biosystems), and 1 µL of first strand cDNA. Reaction conditions were 50 °C for 2 min, 95 °C for 3 min, 35 cycles at 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 60 s. Each assay consisted of three biological and three technical replicates. Each biological replicate represented a pool of 10 individual plants. To confirm the specificities of primers, melting curve analysis and electrophoresis in a 1 % agarose gel of the amplicon were performed.

6.3. Results

6.3.1. Isolation and identification of genes for shikimate and phenylpropanoid biosynthesis enzymes and PR proteins

In the present study, 16 target genes were isolated from the previous study of the *A. koa* transcriptome analysis (Ishihara et al. 2015). They included genes for 1 PR protein (Bgl), 3 enzymes involved in the shikimate biosynthesis pathway (CS, chorismate synthase;

AS, anthranilate synthase; CM, chorismate mutase), and 12 enzymes involved in the phenylpropanoid biosynthesis pathway (PAL; phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, *p*-coumarate:CoA ligase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; CCR, cinnamoyl CoA reductase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl CoA 3-O-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid O-methyltransferase; F5H, ferulate 5-hydroxylase; CHS, chalcone synthase; DFR, dihydroflavonol-4-reductase). Through reverse transcription PCR (RT-PCR) and Sanger sequencing, the sequences for the full ORF were verified and deposited to NCBI database (Table 11). Two PR genes of class I and III chitinases (*Akchit1a* and *Akchit3*, respectively) previously identified by Rushanaedy et al. (2012) were also used for expression analysis in the present study. The BLASTX analysis showed that all the 18 deduced amino acid sequences of the full ORF had 72-98% homologies with protein sequences in other leguminous plants in the NCBI database (Table 11).

Table 11. Genes for shikimate/phenylpropanoid biosynthesis enzymes, pathogenesis-related proteins, and mechanically inducible proteins

GenBank accession no.	Name	ORF length (bp)	BLASTX analysis		
			% identity	Species	GenBank accession no.
Genes for aromatic compound biosynthesis proteins					
KX784950	Chorismate synthase	1305	88	*Glycine max*	XP_003556230.1
KX784951	Chorismate mutase	954	72	*Glycine max*	XP_006603892.1
KX784949	Anthranilate synthase alpha subunit 2	1746	85	*Arachis ipaensis*	XP_016174762.1
Genes for phenylpropanoid biosynthesis proteins					
KX784934	Phenylalanine ammonia-lyase	2151	90	*Glycine max*	NP_001236956.1
KX784935	Cinnamate 4-hydroxylase	1614	89	*Cajanus cajan*	KYP69110.1
KX784937	*p*-coumarate:CoA ligase	1635	92	*L. leucocephala*	ACI23349.1
KX784936	Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase	1314	94	*L. leucocephala*	AGA20364.1
KX784938	Cinnamoyl CoA reductase	1017	93	*L. leucocephala*	CAK22319.1
KX784939	*p*-coumarate 3-hydroxylase	1527	91	*Caragana korshinskii*	AEV93473.1
KX784942	Caffeoyl-CoA O-methyltransferase	735	98	*Leucaena leucocephala*	ABE60812.1
KX784941	Cinnamyl alcohol dehydrogenase	1077	97	*Acacia auriculiformis x Acacia mangium*	ABX75855.1
KX784944	Caffeic acid 3-O-methyltransferase	1098	98	*Acacia auriculiformis x Acacia mangium*	AAY86361.1
KX784940	Ferule 5-hydroxylase	1557	89	*L. leucocephala*	ABS53040.1
KX784933	Chalcone synthase	1170	96	*Cajanus cajan*	KYP67775.1
KX784948	Dihydroflavonol-4-reductase	1038	83	*Glycine max*	BAO53727.1
Genes for pathogenesis-related proteins					
KX784945	β-1,3-glucanase	1116	75	*Sesbania rostrata*	BAE53382.1
JQ677143	Class I chitinase*	972	93	*Acacia mangium*	BAO45893.1
JQ677146	Class III chitinase*	819	73	*Medicago truncatula*	XP_003592154.1
Genes induced by non-wounding mechanical stress					
KX784946	Zinc finger protein	723	55	*G. max*	XP_003555988.1
KX784947	RING finger protein	864	57	*Fragaria vesca*	XP_004296864.1

*ORF and primer sequences for chitinases were obtained from Rushanaedy et al. (2012).

6.3.2. Mechanical stress enhances production of phenylpropanoids and induces genes for PR proteins

The NanoString nCounter and qRT-PCR analyses showed relative expression levels of genes involved in phenylpropanoid biosynthesis at different time points (2, 4, 6, 12, 24, and 48 h) following the mechanical stress treatment. The previous study of mechanical stress in *A. koa* presented no changes in the expression levels of these genes within 1 h of the stress treatment although there was an increase in anthocyanin and lignin contents following 2 weeks of mechanical treatment. Therefore, responses after ≥ 2 h following the stress treatment were analyzed in this present study. At 2 h and 4 h, there were no significant changes in the expression of the genes involved in phenylpropanoid biosynthesis (data not shown); however, at 6 h following the treatment, the stressed *A. koa* seedlings showed a significant increase in expression of 8 genes for CS, PAL, C4H, 4CL, HCT, CCR, and CCoAOMT ($p < 0.05$ for CS, and $p < 0.01$ for the rest; Fig. 23A). They were upregulated by at least 3.9-fold, and C4H was the most induced gene, upregulated by over 90-fold. The other tested genes in the shikimate and phenylpropanoid pathways did not show any change in expression levels (data not shown). At 12 h and 24 h, the expression levels of the genes were either at or below normal levels, except for C4H. The expression levels of all the genes returned to normal levels at 48 h following the stress treatment (Fig. 24).

Following three weeks of daily mechanical perturbations, the total phenolic content of the stressed *A. koa* stems was significantly enhanced compared to the unstressed control (approximately 26% increase; Fig. 23B). The *A. koa* stem extracts were also tested for its antifungal property against *F. oxysporum* f. sp. *koae* through a disk diffusion assay with four biological and three technical replicates, where each biological replicate represented five individual seedlings. The zone of inhibition was formed around disks containing extracts of both stressed and unstressed *A. koa* stems; however, the average radius of the inhibition zone was significantly larger for the stressed *A. koa* extracts (144.6 ± 2.8 mm; Fig. 23C and E), compared to the unstressed control extracts (120.4 ± 2.7 mm; Fig. 23C and F), showing increased antifungal activities ($p < 0.001$; Fig. 23C-F).

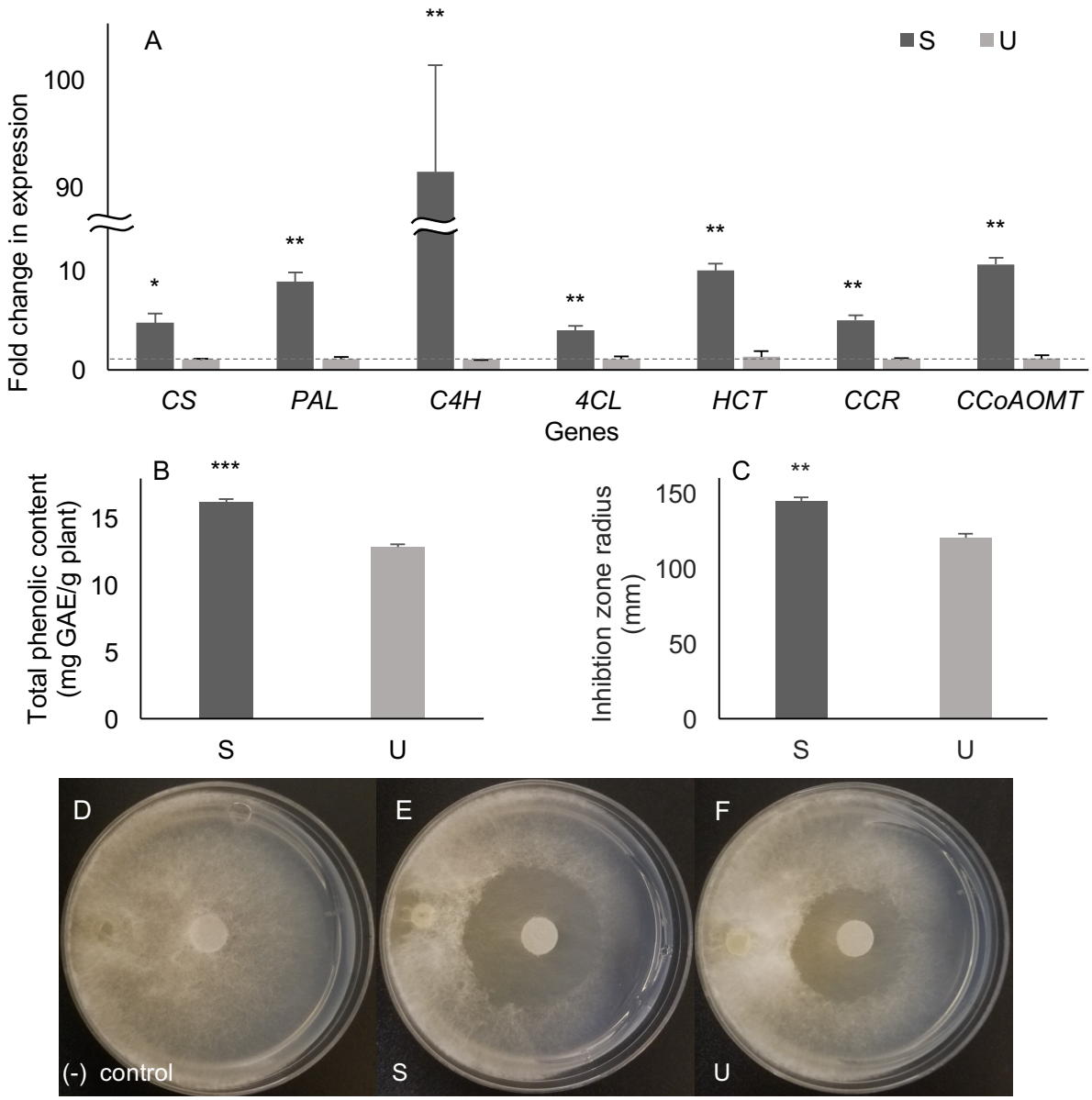

Figure 23. Mechanical stress induces biosynthesis of phenylpropanoids in *Acacia koa*. (A) Fold change in expression of genes involved in the phenylpropanoid biosynthesis in stems at 6 h following mechanical treatment. Fold changes were calculated relative to the unstressed control, indicated by the dashed line at 1-fold. Expression levels were normalized using a reference gene 5.8S rRNA because it showed the lowest stability value among the five candidate genes (*ef1α*, β-actin, 18S rRNA, 5.8S rRNA, ubiquitin-5) in the NormFinder analysis. (B) Total phenolic content in mg gallic acid equivalents (GAE) per gram of *A. koa* stems following 3 weeks of mechanical treatment. (C) The radii of the inhibition zone formed around discs containing *A. koa* stem extracts on Richard's agar media. (D-E) Visual comparison of the inhibition zones around disks containing (D) 70/30 % v/v methanol and 0.1 % v/v aq. formic acid as a negative control, (E) extracts from stressed stems, and (F) extracts from unstressed stems. Abbreviations: CS, chorismate synthase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, *p*-coumarate:CoA ligase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; CCR, cinnamoyl CoA reductase; CCoAOMT, Caffeoyl-CoA O-methyltransferase. U, unstressed control; S, mechanically stressed. Error bars indicate ± SE (n = 3 for each of the stressed and unstressed group, where n represents a pool of 10 seedlings). An asterisk (*) indicates a significant increase in mechanically stressed *A. koa* with $p < 0.05$, two asterisks (**) with $p < 0.01$, and three asterisks (***) with $p < 0.001$.

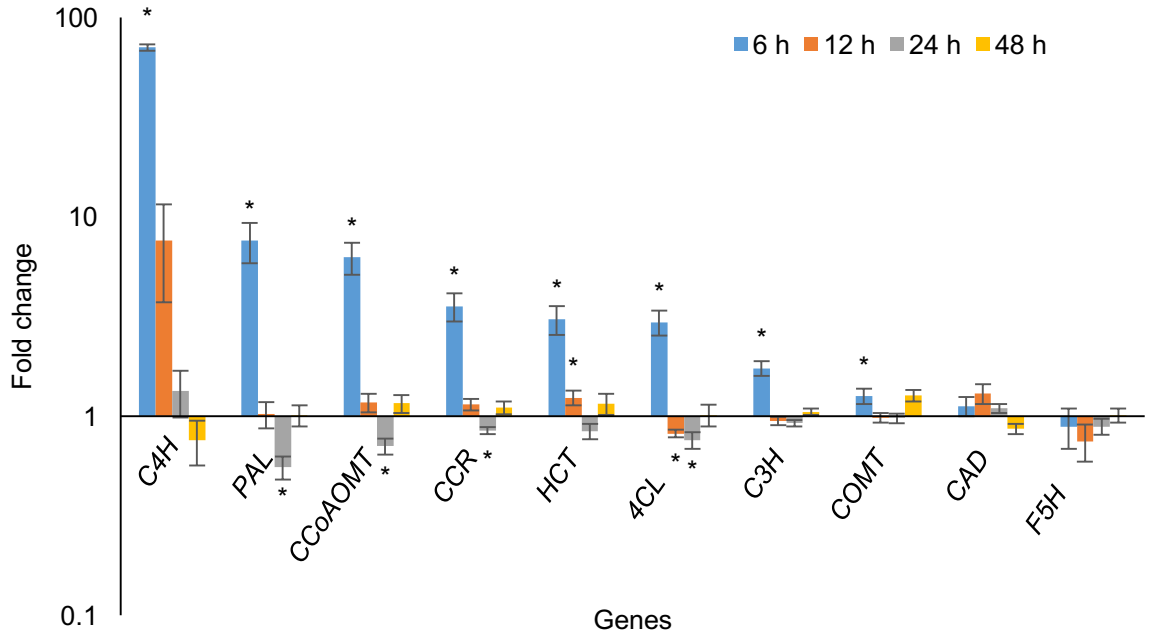

Figure 24. Relative expression levels of the phenylpropanoid biosynthesis genes in *A. koa* at 6, 12, 24, and 48 h following the non-wounding mechanical stimulation in *Acacia koa*. The fold changes in gene expression levels were determined by comparing the stressed and unstressed plants at each time point through the NanoString nCounter and compared at each time point to determine the fold change. Three biological replicates, each consisting of 10 individual *A. koa* seedlings, were analyzed for each time point. An asterisk (*) indicates a significant difference in the stressed plants compared to the expression levels of the unstressed control at each time point with $p < 0.05$. Abbreviations: C4H, cinnamate 4-hydoxylase; PAL, phenylalanine ammonia-lyase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; 4CL, *p*-coumarate:CoA ligase; C3H, *p*-coumarate 3-hydroxylase; COMT, caffeic acid O-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; F5H, ferulate 5-hydroxylase.

Also, the HPLC analysis of the extracts showed that one of the peaks was approximately 23% higher in the extracts from the stressed *A. koa* plants compared to the stressed control ($p < 0.01$; Fig. 25A). The UV spectrum of the compound obtained from the HPLC analysis had a strong peak with a maximum at ~226 nm and three overlapping peaks with maxima ~276, ~278, and ~286 nm, which is a characteristic of substituted indoles (Remers 1972; Fig. 25B). Through the MS analyses, the peak was found to contain a compound with m/z ~291. Consistent with the UV spectrum, the MS/MS spectrums had the general characteristics of substituted indoles, such as fragments at ~130 and ~160 m/z (Rodriguez-Cruz 2005; Fig. 25C). The compound was identified to be malonyl-tryptophan since the fragmentation pattern of the malonyl-tryptophan was identical to the obtained pattern of the plant compound (Fig. 25C). Also, a published mass spectrum of malonyl-tryptophan was highly similar to the spectrum of the obtained compound (Yu et al. 2014).

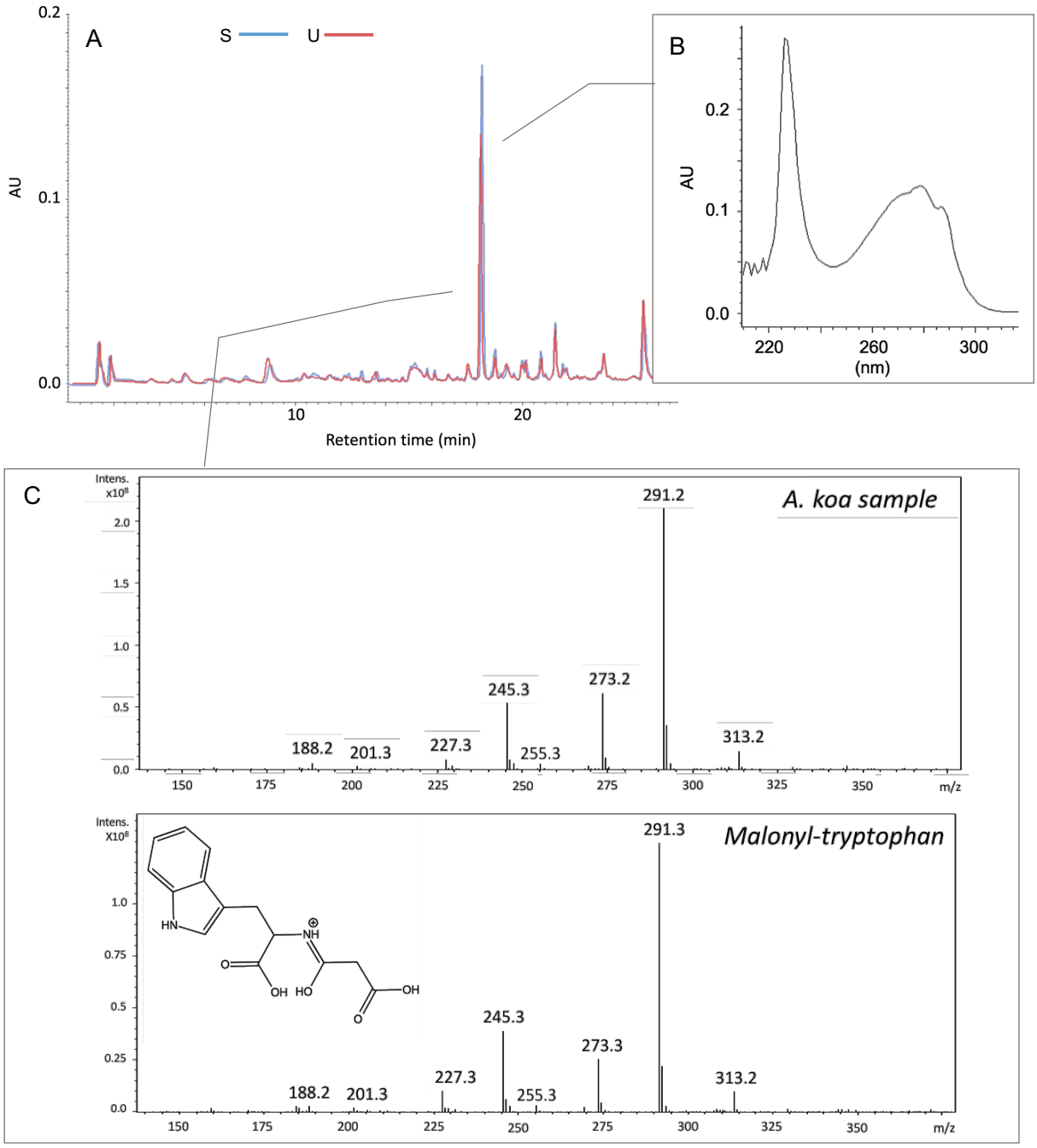

Figure 25. Reverse-phase liquid chromatography (RP-HPLC) and tandem mass spectrometry (MS/MS) analyses of methanolic extracts of *Acacia koa* stems. (A) RP- HPLC chromatograms of the mechanically stressed (blue) and unstressed *A. koa* extracts. The peak at 18 min is significantly higher in the stressed compared to the unstressed ($p < 0.01$, $n = 5$ for the stressed and $n = 3$ for the unstressed). (B) The UV absorption spectrum of the compound at 18 min. There are peaks with a maximum at ~226 nm and three overlapping peaks with maxima ~276, ~278, and ~286 nm, which is a characteristic of indoles. (C) The MS/MS spectrum of the compound at 18 min and malonyl-tryptophan, showing identical fragmentation patterns. Using malonyl tryptophan as a reference standard, the stressed had 3.2 ± 0.13 mg per gram fresh weight (mg/gfw) while the unstressed had 2.6 ± 0.07 mg/gfw.

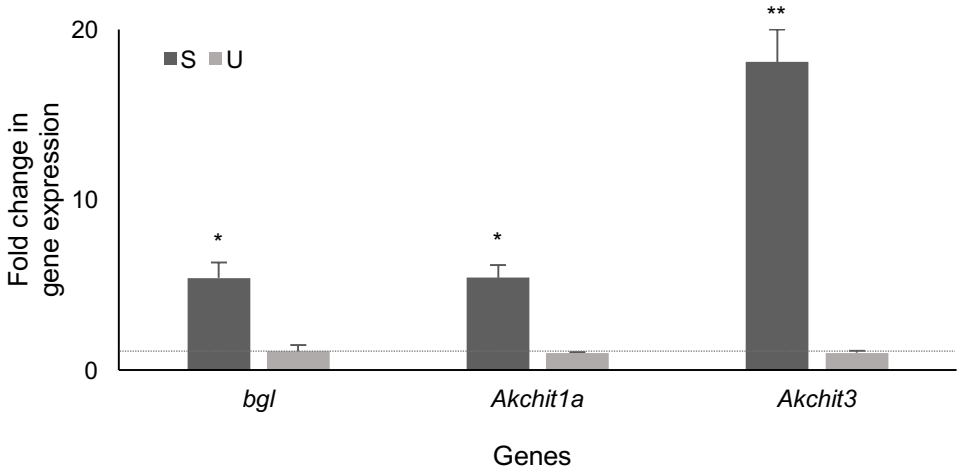

Figure 26. Relative expression levels of genes for antifungal enzymes in *Acacia koa* at 6 h following mechanical stress. Fold changes were calculated relative to the unstressed control, indicated by the dotted line at 1-fold. Expression levels was normalized using a reference gene 5.8S rRNA because the gene showed the lowest stability value among the five candidate genes (*ef1α*, β-actin, 18S rRNA, 5.8S rRNA, ubiquitin-5) in the NormFinder analysis. Abbreviations: *Bgl*, β-1,3,glucanase; *Akchit1a*, class I chitinase; *Akchit3*, class III chitinase; S, mechanically stressed; U, unstressed control. Error bars indicate ± SE (n = 3, where n represents a pool of 10 *A. koa* seedlings). An asterisk (*) indicates a significant increase in mechanically stressed *A. koa* with $p < 0.05$ and two asterisks (**) with $p < 0.01$.

In order to determine if pathogenesis-related proteins (PR) were also involved in the response to mechanical stress, the expression levels of *Bgl, Akchit1a,* and *Akchit3* were analyzed through the qRT-PCR analysis. The results showed that all the three genes were significantly upregulated in the stressed *A. koa* seedlings at 6 h following the mechanical treatment ($p < 0.05$; Fig. 26). The genes *Bgl* and *Akchit1a* were both expressed 5.4-fold higher in the stressed, and the gene *Akchit3* was upregulated over 18-fold, compared to the unstressed control.

6.3.3. Mechanically stressed *A. koa* seedlings are more tolerant to *F. oxysporum* infection and drought stress

Resistance against *F. oxysporum* f. sp. *koae* in mechanically stressed and unstressed *A. koa* was evaluated by growing them in soils inoculated with a mixture of virulent strains of the pathogen. Following 3 separate 100-day inoculation trials, the stressed *A. koa* seedlings showed a significantly higher average survival rate compared to the unstressed control (~2.4-fold increase), where many plants wilted and died within 50 days (Fig. 27A and B). The stressed seedlings also had a significantly higher survival rate following a 50-day drought treatment with PEG6000 (~2.8-fold increase), showing increased drought tolerance compared to the unstressed control plants (Fig. 27C).

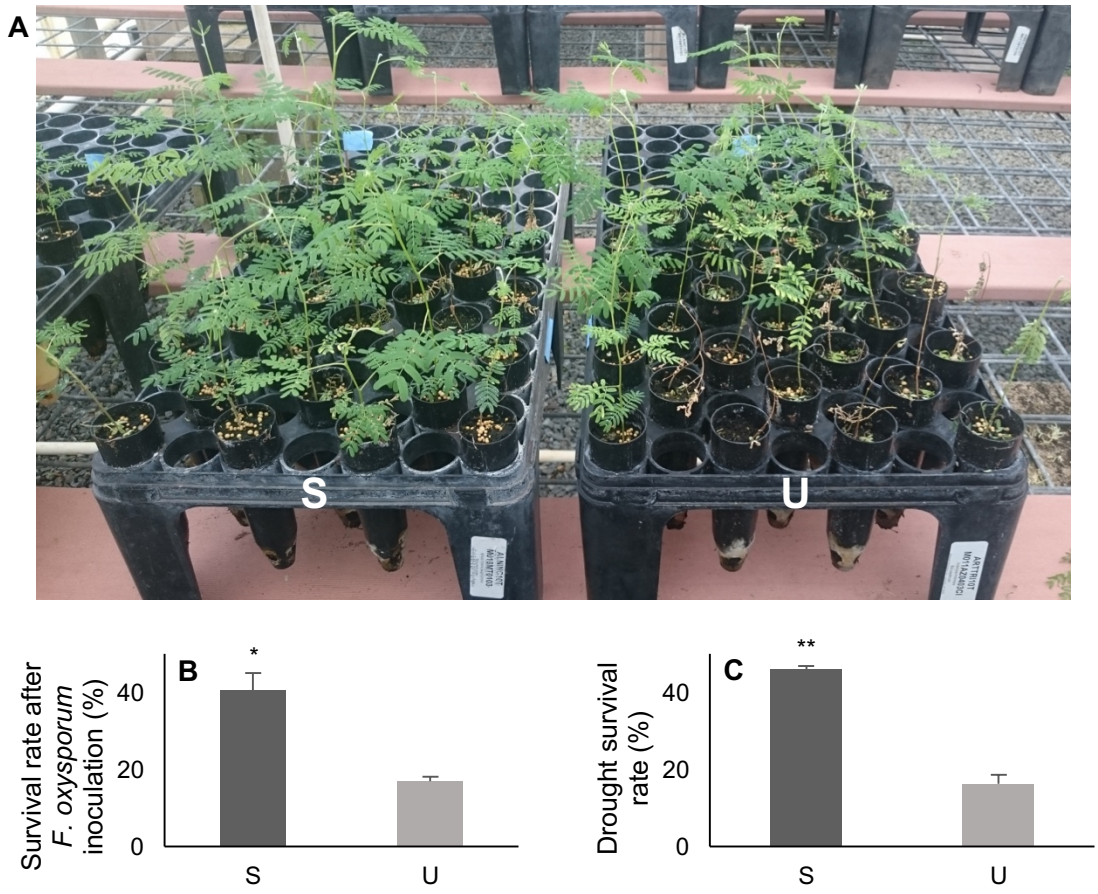

Figure 27. Survival rates of mechanically stressed and unstressed *Acacia koa* plants following (A-B) inoculation by *Fusarium oxysporum* f. sp. *koae* and (C) induction of drought by PEG6000. The stressed and unstressed plants are labeled as S and U, respectively. *Acacia koa* seedlings were mechanically treated for three weeks prior to the following stress trials. (A) Visual mortality difference between mechanically stressed and unstressed *A. koa* in response to *F. oxysporum* infection. (B) Survival rate following 100 days of *F. oxysporum* inoculation in stressed and unstressed plants. (C) Survival rate following 50 days of drought treatment with 15% PEG6000 in stressed and unstressed control. Error bars indicate ± SE (n = 3 trials. Each inoculation and drought trial consisted of 25-35 and 15-20 individual *A. koa* seedlings, respectively). An asterisk (*) indicates a significant increase in mechanically stressed *A. koa* with $p < 0.05$ and two asterisks (**) with $p < 0.01$.

The presence or absence of *F. oxysporum* in the stems was determined through cutting out ~2-mm stem pieces at 2.5 cm from the stem base of inoculated *A. koa* and placing the surface-sterilized stem pieces on Komada agar medium, semi-selective media for *F. oxysporum*. As a negative control, *A. koa* seedlings without inoculation were also tested. *Fusarium oxysporum* was detected in stems of the unstressed plants within one week of inoculation, while it was detected only after two weeks of inoculation in the stressed seedlings (Table 12). The qualitative RT-PCR of the *A. koa* stem RNA with primers specific to a *F. oxysporum* ITS region produced consistent results; *F. oxysporum* was detected in the

stems of the mechanically stressed plants at a later time point following the inoculation (Table 12), indicating that mechanical treatment slowed down progression of *F. oxysporum* infection in *A. koa. Fusarium oxysporum* was also detected in the stems of survived *A. koa* seedlings, both stressed and unstressed, following the 100-day inoculation trial (data not shown).

Table 12. Detection of *Fusarium oxysporum* in stems (piece of 2 mm of stems cut at 3 cm above the stem base) of mechanically stressed and unstressed *Acacia koa* seedlings using qualitative real-time PCR and Komada agar medium

Post inoculation time (week)	RT-PCR		Komada agar medium	
	Stressed	Unstressed	Stressed	Unstressed
1	-	+	-	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+

Note: Three samples were analyzed for each time point. +, detected; -, not detected in all the samples tested

6.3.4. Mechanically stressed *A. koa* seedlings have enhanced expression of genes for antifungal enzymes in response to *F. oxysporum* f. sp. *koae* infection

Through qRT-PCR analysis, the expression levels of the three genes for PR proteins following *F. oxysporum* f. sp. *koae* inoculation were determined in the mechanically stressed and unstressed *A. koa* seedlings at different time points (Fig. 28). At 1 wpi, the expression levels of all the three genes doubled in the unstressed inoculated plants, while there was no significant change in the expression in the stressed inoculated plants. In the unstressed inoculated plants, the expression levels of the genes remained relatively constant at about 2-fold increase, compared to the unstressed uninoculated plants, over four weeks of inoculation; on the other hand, the expression levels fluctuated in the stressed inoculated plants. In these plants, the expression level of *Akchit3* elevated over 6-fold at 2 and 3 wpi, and the expression levels of *Bgl* and *Akchit1a* were also increased approximately 5-fold and 7-fold, respectively. At 4 wpi, the expression levels of the genes in the stressed inoculated *A. koa* were reduced to about the same levels of the unstressed inoculated plants. These results show that mechanical stress induces *A. koa* seedlings to have stronger expression of the PR genes in response to infection by *F. oxysporum* f. sp. *koae*.

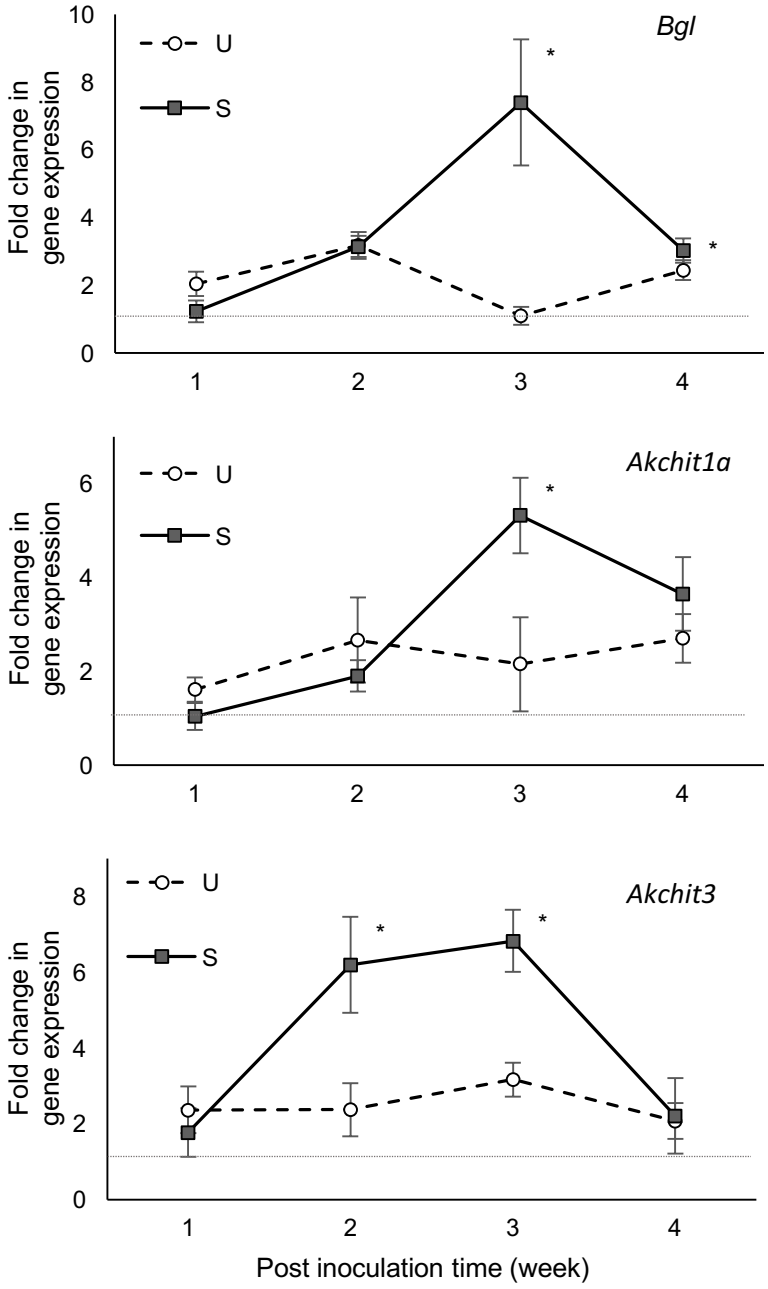

Figure 28. Relative expression levels of genes for antifungal enzymes in mechanically stressed and unstressed *Acacia koa* following *Fusarium oxysporum* inoculation. *A. koa* seedlings were mechanically treated for 3 weeks prior to *F. oxysporum* inoculation. The fold changes in the expression levels of the genes were determined by comparing the inoculated *A. koa* with the uninoculated *A. koa* for each of the stressed (S) and unstressed (U) groups. The dotted line at 1-fold change indicates the expression levels of uninoculated controls. Expression levels were normalized using reference genes ef1α and ubiquitin-5 for week 1, ef1α and 18S rRNA for week 2, 18S rRNA for week 3, and 18S rRNA and ubiquitin-5 for week 4. The reference genes were selected among five genes (*ef1α*, β-actin, 18S rRNA, 5.8S rRNA, ubiquitin-5) based on their stability values obtained from the NormFinder analysis. Error bars indicate ± SE (n = 3, where n represents 10 *A. koa* seedlings). Abbreviations: *Bgl*, β-1,3,glucanase; *Akchit1a*, class II chitinase; *Akchit3*, class III chitinase. An asterisk (*) indicates a significant increase in fold change in mechanically stressed *A. koa* compared to unstressed *A. koa* following the inoculation, with $p < 0.05$.

The genes for one shikimate and three phenylpropanoid biosynthesis enzymes were similarly highly expressed in the stressed *A. koa* in response to the inoculation by *F. oxysporum* f. sp. *koae.* At 1-3 wpi, there was no significant induction of the genes for the phenylpropanoid biosynthesis enzymes in either stressed or unstressed inoculated *A. koa* plants (data not shown). However, at 4 wpi, the expression of CS and CHS were elevated in both stressed and unstressed inoculated plants, and the expression level of CS was significantly higher in the stressed inoculated *A. koa* than the unstressed inoculated (Fig. 29). Also, the genes for PAL, and DFR were induced by *F. oxysporum* f. sp. *koae* infection at 4 wpi, only in the stressed seedlings. The gene for the zinc finger protein (ZFP) was also upregulated in the stressed, inoculated plants, whereas the gene for the RING finger protein (RING) was induced in both stressed and unstressed plants upon *F. oxysporum* infection.

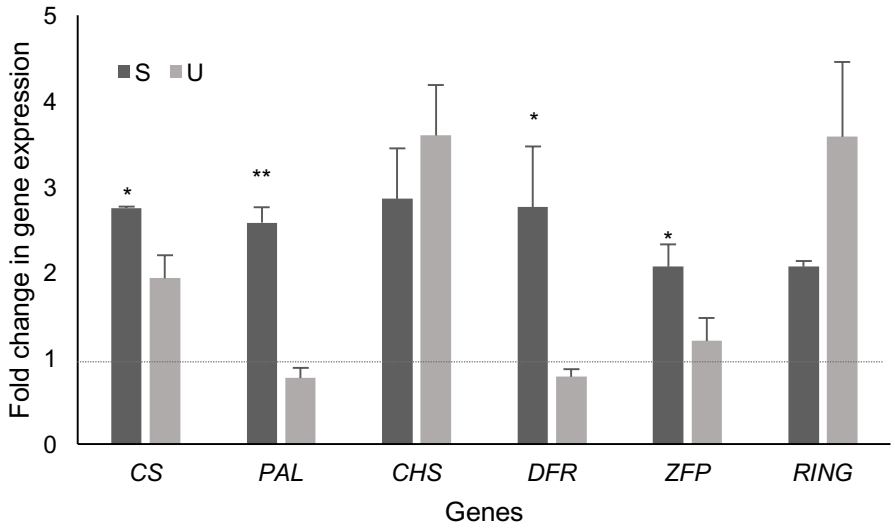

Figure 29. Relative expression levels of shikimate/phenylpropanoid biosynthesis genes and mechanically inducible genes in mechanically stressed and unstressed *Acacia koa* following *Fusarium oxysporum* inoculation. *A. koa* seedlings were mechanically treated for 3 weeks prior to *F. oxysporum* inoculation. The fold changes in the expression levels of the genes were determined by comparing the inoculated *A. koa* with the uninoculated *A. koa* for each of the stressed (S) and unstressed (U) group. The dotted line at 1-fold change indicates the expression levels of uninoculated controls. Reference genes were selected among five genes (*ef1α*, β-actin, 18S rRNA, 5.8S rRNA, ubiquitin-5) based on their stability values obtained from the NormFinder analysis. Expression levels were normalized using the selected reference genes 18S rRNA and ubiquitin-5. Error bars indicate ± SE (n = 3, where n represents 10 *A. koa* seedlings). An asterisk (*) indicates a significant increase in gene expression fold change in mechanically stressed *A. koa* compared to the unstressed control following the inoculation, with $p < 0.05$, and two asterisks (**) $p < 0.01$. Abbreviations: CS, chorismate synthase; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; DFR, dihydroflavonol-4-reductase; ZFP, zinc finger protein; RING, RING finger protein.

6.4. DISCUSSION

Results from the previous study of mechanical stress in *A. koa* suggested that mechanical stimulation may induce tolerance to pathogens because genes for various enzymes involved in disease resistance, such as NBS-LRR proteins and RING finger ubiquitin ligase, were highly upregulated immediately following the mechanical stress. In the present study, this idea of induced disease resistance by mechanical stress was tested with *F. oxysporum* f. sp. *koae,* a fungal pathogen that causes a devastating wilt disease in *A. koa*. To minimize physical variables that may affect the results, a period of three weeks was selected for mechanical treatment prior to the fungal inoculation as there was no significant difference in plant height between the stressed and unstressed *A. koa* seedlings at this time point (no data shown). This study showed that the survival rate increased more than two-fold for the *A. koa* seedlings that were mechanically stressed prior to the inoculation of *F. oxysporum* f. sp. *koae* compared to the control seedlings that did not receive mechanical stress. It indicates that mechanical stimulation indeed enhanced tolerance to *F. oxysporum* f. sp. *koae* in *A. koa.*

Disease resistance is known to be induced by wounding treatments (for review, see Savatin et al. 2014); however, it can be also induced by non-wounding mechanical treatments. For instance, smaller lesion areas were observed on the detached *Arabidopsis* leaves when they were infected with *Botrytis cinerea* immediately following the sweeping of the leaf surface of *Arabidopsis* (Benikhlef et al. 2013). Similarly, detached leaves of *Phaseolus vulgaris* previously exposed to fan-produced wind showed a greater degree of resistance against infection by *Colletotrichum lindemuthianum* (Cipollini 1997). On the contrary, Shawish and Baker (1982) showed that gentle shaking of the stems for 1 min made *Solanum lycopersicum* (*Lycopersicon esculentum*)*, Linum usitatissimum,* and *Pisum sativum more* susceptible to the infection by *F. oxysporum.* Whether a mechanical stimulus makes plants more or less resistant may be decided by the amount of mechanical treatment because *Oryza sativa* plants were severely affected by leaf and panicle blast when they received fan-induced wind at a velocity > 7.3 m/s for 30 min, while they were not affected as much when the velocity was below 7.3 m/s (Taguchi et al. 2014). In the present study, the mechanical treatment was few-second gentle bending of stems in four cardinal directions once a day for three weeks prior to the fungal inoculation. Even with such a small amount of mechanical treatment, it gave *A. koa* plants prolonged disease resistance, helping more than twice the number of seedlings survive in 100-day inoculation trials. Further analysis indicated that *F. oxysporum* wilt resistance may be induced due to elevated drought

tolerance, increased phenylpropanoid biosynthesis, and priming of pathogenesis-related proteins in the mechanically stressed *A. koa*

6.4.1. Enhanced wilt-disease resistance due to increased drought tolerance in mechanically stressed *A. koa*

Soil-borne *F. oxysporum* typically enters the host plants through the root system and advances intercellularly to the plant's xylem vessels (MacHardy and Beckmann 1981). The fungus is then able to spread upwards through the vascular system, leading to clogging of vessels due to the accumulation of mycelium and formation of host plant defense compounds, such as tyloses and gum (Pietro et al. 2003). As a result, the flow of water to the upper regions of the plant is disrupted, thereby causing the plant to have wilt-related symptoms. Since the disruption of water movement is the major cause of the wilt disease, there should be close relationship between resistance to the vascular wilt disease and tolerance to drought. For example, several studies showed that the leaf surface temperature can be used to access the levels of resistance against fungal wilt diseases and drought. In response to water shortage, plants generally reduce the transpiration rate, which leads to an increase in leaf surface temperature (Rispail and Rubiales 2015). Increase in the leaf temperature were observed in both wilt and drought susceptible plants in response to fungal or drought treatment compared to resistant plants (Hirayama et al. 2006; Rispail and Rubiales 2015). Similarly, a vascular-wilt-disease resistant *Arabidopsis* mutant was found to be also drought tolerant (Yadeta et al. 2014).

In the present study, the mechanically stressed *A. koa* seedlings exhibited increased drought tolerance in addition to increased *Fusarium* wilt resistance. Many transcription factors involved in drought stress, such as WRKYs, MYBs, NACs, DREBs, and ZFP, were upregulated quickly in response to the mechanical stress (Lata and Prasad 2011; Nuruzzaman et al. 2013; Ambawat et al. 2013; Bakshi and Oelmüller 2014), which may lead to the enhancement of drought tolerance in the stressed plants. This ability to withstand disrupted water flow in the xylem vessels is most likely one of the important characteristics for wilt-disease resistance.

6.4.2. Elevated phenylpropanoid accumulation and antifungal activities in the extract of mechanically stressed *A. koa*

This study showed that the total phenolic content was elevated in the mechanically stressed *A. koa* seedlings because of upregulation of some of the genes involved in the

shikimate and phenylpropanoid biosynthesis pathways (Fig. 30). One of these mechanically induced genes encodes CS, an enzyme that generates chorismate, which is an important precursor molecule for synthesis of aromatic compounds, including tyrosine and phenylalanine. Although phenylalanine is the initial molecule of the phenylpropanoid biosynthesis pathway, there was no significant upregulation of CM, which converts chorismate to phenylalanine precursors (Fig. 30). The increased CS expression level is thus most likely one of the reasons that the mechanically stressed *A. koa* had an increased level of the total phenolic content.

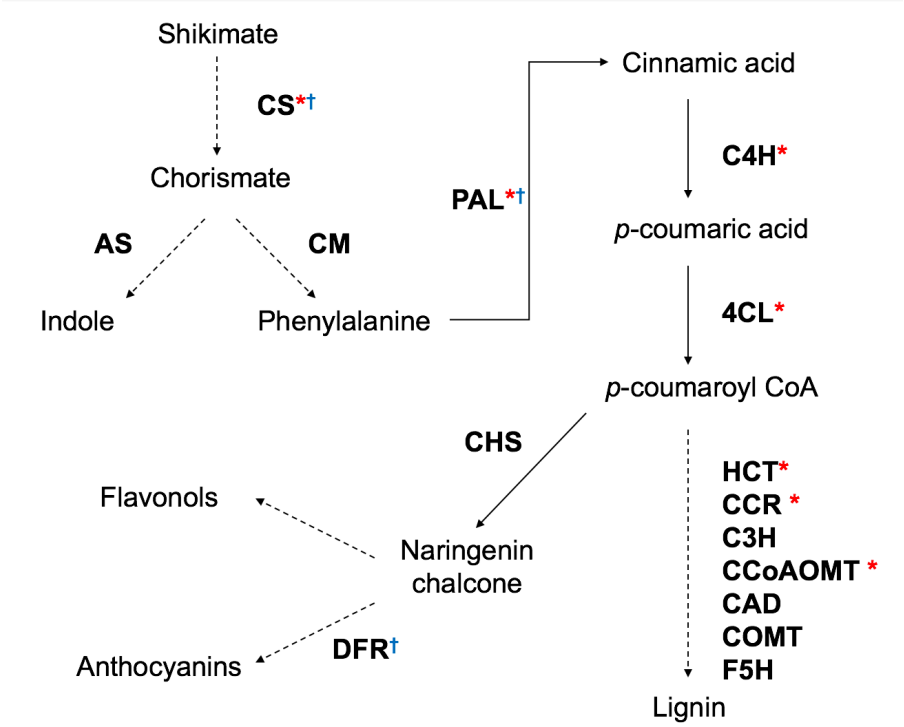

Figure 30. Simplified scheme for phenylpropanoid biosynthesis. The initial molecule phenylalanine in the phenylpropanoid pathway is derived from chorismate synthesized in the shikimate pathway. The phenylpropanoid biosynthesis pathway produces wide varieties of secondary metabolites, including lignin, flavonols, and anthocyanins. An asterisk (*) indicates that genes for the enzymes were induced by mechanical stress in *A. koa*. A dagger (†) indicates that genes for the enzymes were expressed more in mechanically stressed *A. koa* in response to *F. oxysporum* infection. Abbreviations: CS, chorismate synthase; CM, chorismate mutase; AS, anthranilate synthase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, *p*-coumarate:CoA ligase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; CCR, cinnamoyl CoA reductase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid 3-O-methyltransferase; F5H, ferule 5-hydroxylase; CHS, chalcone synthase; DFR, dihydroflavonol-4-reductase.

The genes for the first three enzymes of the phenylpropanoid biosynthesis pathway PAL, C4H, and 4CL were also upregulated in the mechanically stressed *A. koa.* These enzymes are important since they produce the major pivotal intermediate of this pathway, *p*-coumaroyl CoA, which is the precursor for a variety of secondary metabolites. The increase in the PAL activity was also observed in other plants, such as *Bryonia dioica* and *S. lycopersicum,* at ≥ 6 h following the mechanical treatment. The genes for enzymes in the monolignol pathways, including HCT, CCoAOMT, and CCR, were also induced by the mechanical stress in *A. koa.* These results are consistent with the increase in lignin content in the stressed plants as described previously. Although the increases in anthocyanin and total phenolic contents were observed in the stressed *A. koa* in this and the previous study, there was no change in the expression levels of the genes for CHS and DFR, the enzymes involved in biosynthesis of flavonoids and anthocyanins. Therefore, as suggested previously, the present study also supported that increased anthocyanins in the stressed *A. koa* were inadvertently produced due to enhancement of the upstream phenylpropanoid and monolignol biosynthesis pathways.

Many phenylpropanoids are known to have antimicrobial activities, and multiple studies have reported that phenylpropanoid biosynthesis is involved in fungal disease resistance in various plants. Multiple studies have shown the involvement of PAL in disease resistance; overexpression of PAL in *Nicotiana tabacum* markedly enhanced resistance to fungal infection by *Cercospora nicotinae* (Shadle et al. 2003), while a PAL-knockout mutant in rice exhibited increased susceptibility to fungal infection by *Magnaporthe oryzae* (Duan et al. 2014). Kamphuis et al. (2012) also observed a significant increase in the transcripts for C4H and 4CL in *Medicago truncatula* in response to the infection with a fungal pathogen *Phoma medicaginis*. It is likely that phenylpropanoid compounds synthesized through these enzymes play an important role in *F. oxysporum* wilt resistance in *A. koa*. In the present study, it was observed that there was a positive correlation between the total phenolic content and the antifungal activity because the extracts of the mechanically stressed *A. koa* containing higher levels of phenolic compounds slowed the growth of *F. oxysporum* f. sp. *koae in vitro* on agar media. Consistently, the progression of the infection by *F. oxysporum* was also delayed *in vivo* in the mechanical stressed *A. koa* as the fungus was detected in the stem of the stressed plants one week after than in the unstressed plants; it is plausible that the enhanced antifungal activities in the stressed plants slowed the invasion by the fungus.

The increased lignin content was also suspected to be one of the factors that slowed the progression of infection in the mechanically stressed *A. koa* because lignin, an important component of the cell wall, could physically hinder the invasion by *F. oxysporum* f. sp. *koae.* However, no significant difference in lignin content was observed between the stressed and the unstressed control plants following three weeks of mechanical treatment (data not shown). The previous study showed increase in lignin content following two months of mechanical treatment, so lignin accumulation in response to mechanical stress appears to occur slowly. There may be technical limitations to quantify minute differences in lignin content, but the results indicate that lignin is not likely the major factor that delayed the progression of infection.

The mechanically stressed *A. koa* also had higher amounts of tryptophan derivatives. In the tryptophan biosynthesis pathway, the enzyme AS catalyzes the first step of converting chorismate to anthranilate, the precursor of tryptophan. However, the expression of the gene for AS did not change in response to the mechanical treatment. Therefore, the elevated amount of the tryptophan derivatives could be also due to the increased CS expression level because it can produce greater amounts of the precursor molecule for AS as well as CM (Fig. 30). Like phenylpropanoids, tryptophan derivatives are known to be involved in disease resistance (Bednarek et al. 2004; Stahl et al. 2016). In this study, the most prominently induced secondary metabolite found in the methanolic plant extracts through the HPLC analysis was a tryptophan derivative. The enhanced tryptophan compound was identified to be malonyl-tryptophan since the MS/MS fragmentation pattern of the plant compound was identical to that of malonyl-tryptophan. The molecule was proposed to be a possible auxin precursor (Rekoslavskaya 1986) and was observed to accumulate in response to drought stress in *S. lycopersicum* (Rekoslavskaya et al. 1992). Although malonyl-tryptophan was identified decades ago (Good and Andreae 1957), there is only a handful of studies for this molecule, and its function still remains unclear. Although antifungal activity of this compound was not observed *in vitro* against *F. oxysporum* (no data shown), it may be an important phytohormone to protect plants from drought stress; therefore, it may also protect from the *Fusarium* wilt, which causes drought-related symptoms.

6.4.3. Priming of PR proteins and shikimate and phenylpropanoid biosynthesis enzymes in the mechanically stressed A. koa

Plants do not have specialized immune cells, but they have their own immune system. It is well-known that plants often develop enhanced disease resistance due to

infection by necrotizing pathogens or even colonization by non-pathogenic microorganisms (for review, see Conrath 2006 and Choudhary et al. 2007). Wounding and treatments by various chemicals, such as salicylic acid (SA) and benzothiadiazole (BTH), can also induce disease resistance for future infection by a wide range of pathogens (Cohen 2002; for review, see Goellner and Conrath 2008). The key mechanism of induced resistance is priming, or enhanced ability to respond effectively in response to biotic or abiotic stresses. Priming agents typically initiate plant defense responses, including accumulation of PR proteins and phenolics, and then sensitize the plants for future attacks. For example, *Arabidopsis* plants primed by wounding showed increase in the antimicrobial molecule camalexin upon the infection with *B. cinerea* (Chassot et al. 2008). Similarly, the expression of *PAL* gene is also shown to be upregulated in the primed state following infection by a pathogen in various plants (Thulke and Conrath 1998; Kohler et al. 2002; Wu et al. 2013; for review, Conrath 2006).

The present study demonstrated that non-wounding mechanical stimulation, such as gentle bending of the stems, can also prime *A. koa* seedlings for defense against infection by *F. oxysporum.* In the prior non-wounding mechanical treatment, the defense genes were induced, priming for systematic induced resistance. At 1 wpi, the expression levels of the PR genes were slightly higher for the inoculated unstressed *A. koa,* which may be because the unstressed plants were already infected by the time while the stressed plants were not. The primed state of the stressed *A. koa* was observed at 2 and 3 wpi; their expression levels of all the three PR genes were enhanced over 5-fold, while the expression levels of the inoculated and unstressed *A. koa* seedlings were upregulated only 2-fold. Rushanaedy et al. (2012) presented that resistant *A. koa* families had higher expression levels of these chitinases upon infection by *F. oxysporum,* compared to susceptible families. Consistently, the present study showed that the expression levels of the PR genes were associated with disease resistance. Also, the expression of *CS*, *PAL*, and *DFR* genes were enhanced in the mechanically treated *A. koa* following *F. oxysporum* inoculation. It is notable that the expression of the *DFR* gene was elevated although it was not induced by the previous mechanical treatment. The upregulation on these genes would elevate the phenolic and indolic contents of the stressed and inoculated *A. koa* seedlings, and the plants would have higher antifungal activities along with the elevated PR proteins.

6.4.3. Conclusion

The non-wounding mechanical stress induced resistance against *F. oxysporum* f. sp. *koae* in *A. koa*. The stressed *A. koa* grew more tolerant to drought stress, produced higher contents of secondary matabolites, and enhanced the ability to respond effectively following the infection by *F. oxysporum.* Therefore, the study implies the importance of mechanical stimulation in the plant defense development, and mechanisms underlying disease resistance against *F. oxysporum* may be studied simply by applying mechanical stresses to *A. koa*.

CHAPTER 7
GENERAL DISCUSSION AND CONCLUSION

Non-wounding mechanical stress produces thigmomorphogenetic phenotype and disease resistance in *A. koa*

Acacia koa, an economically important timber-wood tree in the Hawaiian Islands, is currently suffering from a destructive wilt and dieback disease caused by a fungal pathogen *Fusarium oxysporum* f. sp. *koae*. Previous studies have shown that stimulation by environmental stresses, including drought and wind, can induce resistance to various pathogens in plants (reviewed in Chapter 2). Non-wounding mechanical stresses, such as gentle wind and touching, have also been shown to enhance disease resistance in some plants (Beniklef et al. 2013; Taguchi et al. 2014; Elsharkawya et al. 2015). Therefore, studying mechanically induced resistant plants may be useful to elucidate how disease resistance is conferred. In this research, the effects of a non-wounding mechanical stress were studied in *A. koa* to determine transcriptional, biochemical, and morphological changes, and levels of induced disease resistance to *F. oxysporum.*

First, because the genomic information of *A. koa* is limited, its whole transcriptome was sequenced and assembled to identify genes that may be related to disease resistance. Prior to this experiment, a modified RNA extraction method was developed to facilitate isolation of high-quality RNA with a sufficient quantity. Through Illumina sequencing, over 88 million high-quality paired-end raw reads were generated. Trinity *de novo* assembly of those reads yielded 85,533 unigenes with an average length of 641 bp. From these assembled unigenes, complete coding sequences for various genes were identified; they include genes for the plant immunity, shikimate pathway, and phenylpropanoid biosynthesis pathway. These sequences were used in the following experiments to analyze differential gene expression in response to non-wounding mechanical stress in *A. koa.*

Effects of a non-wounding mechanical stress were studied in three different ways: (i) immediate gene expression following the stress treatment, (ii) biochemical changes following 8 weeks of daily treatment, and (iii) morphological changes following 8 and 24 weeks of daily stress treatment. To identify genes induced by mechanical stress, 4,000 genes that may be related to disease resistance, and growth and development were selected from the transcriptome data and used for a microarray analysis. The upregulated genes were verified by nCounter and qRT-PCR analyses. For a mechanical stress treatment, *A. koa* seedlings were gently bent once in each of the four cardinal directions. Within 10-60 min following the

treatment, over 50 genes were induced more than two-fold by the mechanical stress, including the genes for calcium signaling, ethylene (ET) biosynthesis, abscisic acid degradation, stress-related transcriptional regulation, and disease resistance (Fig. 31A). Also, morphological and biochemical changes due to a prolonged mechanical stress were studied. Following 2-6 months of daily non-wounding mechanical treatment, the stressed *A. koa* had significantly reduced stem length whereas they had significantly increased stem diameter, number of the xylem cells, and accumulation of phenolic compounds, including anthocyanin and lignin, which are often induced in defense responses (Fig. 31B).

Based on the observations that the *A. koa* treated with non-wounding mechanical stress had higher expression of several genes involved in disease resistance and increased levels of phenolic compounds, it was hypothesized that mechanically stressed *A. koa* plants have increased disease resistance against infection by *F. oxysporum* f. sp. *koae.* The results of 100-day trials of *F. oxysporum* inoculation showed that the survival rate doubled in the mechanically stressed *A. koa.* The plant extract of the mechanically stressed *A. koa* had higher total phenolic content and stronger antifungal activities, so it may have slowed down the fungal growth. The stressed plants also showed enhanced expression levels of the genes for shikimate and phenylpropanoid biosynthesis enzymes and pathogenesis-related (PR) proteins upon infection by *F. oxysporum* f. sp. *koae,* which suggests that the non-wounding mechanical stress can prime *A. koa* plants for defense against the fungal pathogen. Many of these genes were also expressed following the mechanical stress alone without infection (Fig. 31A). Besides the PR genes and phenylpropanoid biosynthesis genes, two of the most upregulated genes induced by the mechanical stress, *ZFP* and *RING,* were also upregulated in the stressed, inoculated plants. These results indicate that the plants had overlapping pathways in response to the mechanical stress and infection.

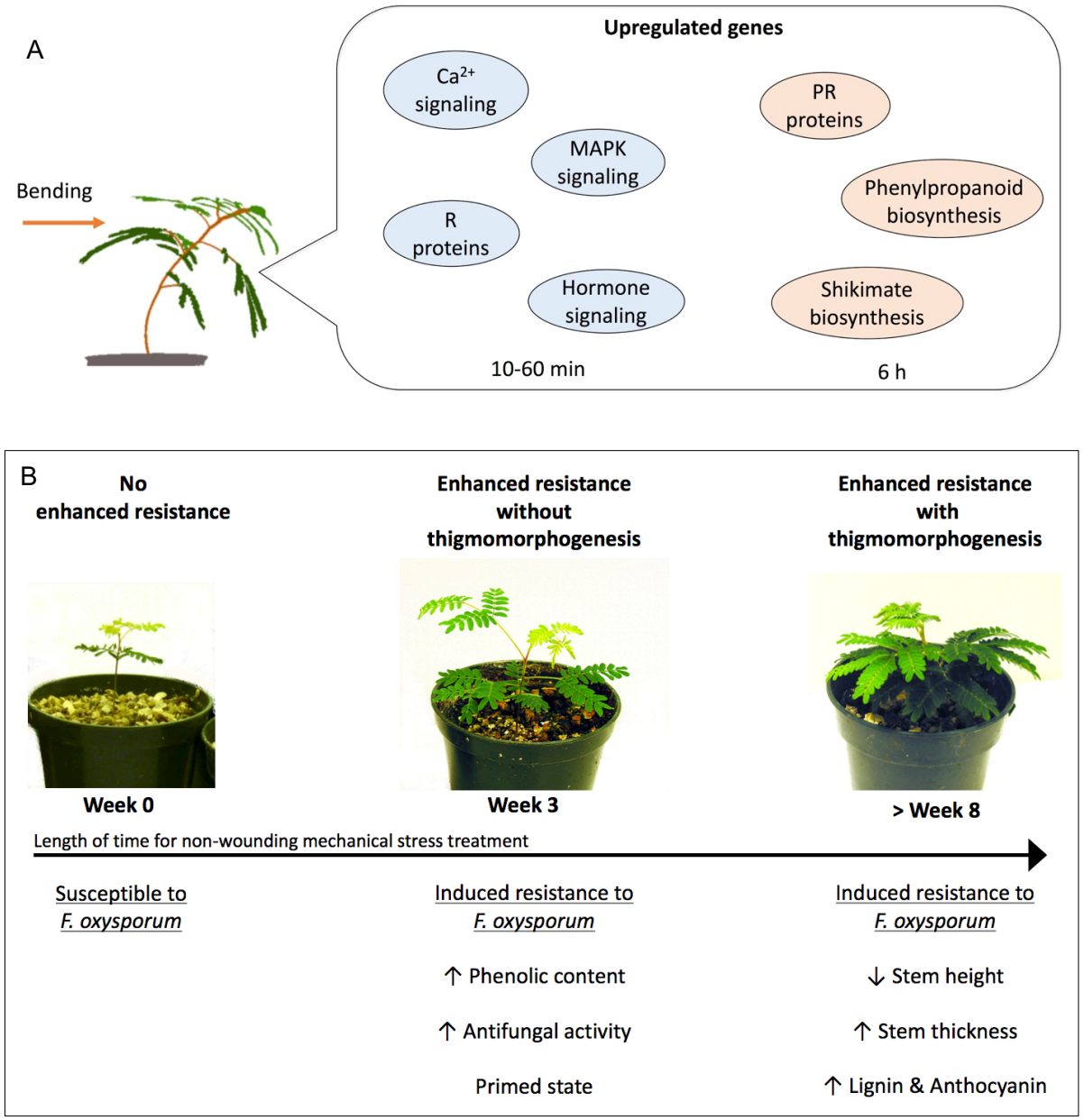

Figure 31. Effects of the non-wounding mechanical stress in *Acacia koa*. (A) Transcriptional changes at 10-60 min and 6 h following the stress treatment. Within 10-60 min of the treatment, genes related to calcium signaling, MAPK signaling, disease resistance (R) proteins, and hormone signaling were upregulated; at 6 h following the stress, genes for pathogenesis-related (PR) proteins and phenylpropanoid and shikimate biosynthesis enzymes. (B) Biochemical and morphological changes were induced by the non-wounding mechanical stress. Following 3 weeks of stress treatment, *A. koa* seedlings had induced resistance against *F. oxysporum* with enhanced total phenolic content and antifungal activity. Following > 8 weeks of stress treatment, thigmomorphogenetic changes were observed as well as increase in lignin and anthocyanin contents.

This study showed that the short-term (3 weeks) non-wounding mechanical stress produced disease resistance whereas the long-term (8-24 weeks) mechanical stress produced the thigmomorphogenetic phenotype of stunned growth in *A. koa* as well as disease resistance (Fig. 31B). Similar observations have been made in other plants treated with different doses of defense elicitors, such as salicylic acid (SA), jasmonic acid (JA), benzothiadiazole (BTH), β-aminobutyric acid (BABA), and disease resistance (R) genes. With low doses of these defense elicitors, resistance was still induced with only minor reductions in growth (Van Hulten et al. 2006); in contrast, when high doses were applied, although resistance was enhanced, plant fitness traits, such as growth and seed production, were reduced in various plants, including *Arabidopsis, Cucumis sativus, Raphanus raphanistrum*, *Nicotiana attenuate*, and *Capsicum annuum* (Agrawal et al. 1999; Baldwin 1998; Van Dam and Baldwin 2001; Romero et al. 2001; Cipollini 2002; Heil 2000; Heidel et al. 2004). Also, *Arabidopsis* mutants with constitutive expression of PR genes or SA synthase resulted in a dwarfing phenotype (Bowling et al. 1994, 1997). Consistently, the *A. koa* seedlings that were mechanically treated for > 8 weeks showed a significantly reduced growth, probably due to constitutive gene expression for defense instead of growth, while the *A. koa* seedlings that were stressed only for three weeks showed normal growth with enhanced disease resistance after the treatment. Based on the immediate transcriptional changes observed in the mechanically stressed *A. koa*, there was overexpression of several genes involved in hormone metabolism and signaling such as abscisic acid 8'-hydroxylase, 3-aminocyclopropane 1-carboxylate synthase (ACS), and ET-responsive transcription factors (Fig. 31A). Therefore, the levels of the hormone defense elicitors could be higher in the stressed *A. koa* seedlings. With the prolonged daily stress treatment, the hormone levels could be constitutively high, leading to the stunted phenotype (Fig. 31B).

Although various studies have shown enhanced disease resistance in a dose-dependent manner (Wiese et al. 2004), an appropriate amount of elicitors should be given so that plants can balance fitness and defense. As observed in this study, *A. koa* seedlings that were mechanically stressed longer may be more resistant with higher accumulation of defense proteins and secondary metabolites; however, they are not desirable due to their reduced growth. On the other hand, the plants with a shorter period of the mechanical treatment were more balanced because they had normal growth and yet were primed for defense against *F. oxysporum*, responding with stronger expression of defense genes following the fungal infection. To grow plants that have both high resistance and high yield, their ability to 'remember' and initiate a more effective and faster defense for future

challenges is valuable because once plants are in the primed state, they can re-allocate their resources from defense to growth and development.

Another question that arises from this study is why *A. koa* trees in the wild, which simultaneously encounter various abiotic and biotic stimuli, do not develop stronger resistance against infection by *F. oxysporum* although gentle bending alone can induce resistance in *A. koa* grown in the green house. One possible reason is the complex interaction among plant responses. In this study, *A. koa* was treated with only one mechanical stress and then infected with *F. oxysporum.* However, *A. koa* trees in nature could be affected by many stimuli, such as drought, wind, wound, and pathogens, at once. Several studies have shown that during simultaneous biotic and abiotic stresses, the responses are not additive because different signaling pathways interact and inhibit one another. For example, numerous genes that were not induced by either heat or drought were up-regulated by a combination of heat and drought stresses in *Arabidopsis* and tobacco (Rizhsky et al. 2002, 2004; for review, see Atkinson and Urwin 2012). Similarly, a unique set of genes were expressed in response to a combination of high light and high temperature in sunflower (Hewezi et al. 2008). Because plants exhibit a complex and differential set of responses, they can grow either susceptible or resistant in response to simultaneous exposure to various stresses. As reviewed in Chapter 2, the interaction of abiotic and biotic responses may still differ depending on the biotic and abiotic elicitors, dosage of elicitors, timing, and perhaps on plant species. Therefore, *A. koa* trees in nature, which are stressed by many factors, perhaps do not have disease resistance to *F. oxysporum* as enhanced as *A. koa* that is given only a non-wounding mechanical stress prior to infection.

Although this research has reached its specific aims, there are some unanswered questions to which this research could not address the answers, and future studies to answer these questions will be important for the *A. koa* improvement program. One of the limitations is that although the plant extract was found to have antifungal activities, no antifungal compounds that were highly accumulated in the stressed plants were identified. The HPLC analyses of the plant extract focused more on the hydrophilic molecules, so there may be antifungal compounds among more lipophilic molecules, or perhaps the antifungal activity may be only due to an increase in overall antioxidant molecules, such as phenolic and indolic compounds. Future studies with more thorough analyses of *A. koa* extracts may help identify an antifungal compound. Also, the plants underwent three weeks of the bending treatment to induce disease resistance in this study, but it did not determine the minimal length of the mechanical treatment to induce disease resistance. There could be other types

of non-wounding mechanical stimuli that would also induce resistance. Moreover, although the mechanically stressed *A. koa* seedlings survived for 100 days following the inoculation by *F. oxysporum*, it was not determined if they would still have induced resistance after 100 days due to time constrains; several more years of observation would be necessary.

In conclusion, the non-wounding mechanical stress through gentle bending of stems for several weeks induced disease resistance against infection by *F. oxysporum* f. sp. *koae* in *A. koa* without affecting the plant growth. Therefore, it is possible to study disease resistance simply by applying non-wounding mechanical stimuli to plants; the mechanically stressed *A. koa* can be used as a model to further elucidate mechanisms underlying disease resistance to *F. oxysporum*. It may contribute toward identifying important genes and molecular factors related to disease resistance, and they can be used as markers for seedling selection in the *A. koa* improvement program. The study showed importance of non-wounding mechanical stimulation in development of disease resistance during early development of *A. koa*, and it may applicable to other woody plants. Dudley et al. (2015) has shown that the screening method through selecting survived *A. koa* seedlings in 100-day *F. oxysporum* inoculation trials is useful to eliminate susceptible plants prior to out-planting; based on several years of field experiments using the screened *A. koa* seedlings for out-planting, there was a significant improvement in the survival rate, particularly at a site in Maunawili, Hawai‘i, where high disease pressure is observed. Therefore, although it is uncertain exactly how long the induced resistance lasts, it is highly possible that mechanically stressed *A. koa* seedlings that survived the 100-day inoculation trials will also survive once out-planted. In future studies, it will be important to determine whether this induced resistance lasts longer in field trials and whether it is a heritable trait in *A. koa* as observed in other plants.

Furthermore, this research contributed to many other research opportunities of *A. koa*. Especially, the transcriptome analysis of this plant species offers valuable sequence resources. For example, based on the transcriptome analysis, two projects were developed in the lab: (i) identification of microsatellite in the actively transcribed genes and (ii) identification of genes for tannin biosynthesis. In addition, so a treasure trove of sequences is left behind; since the sequences have been deposited in online database, any important gene sequences can be identified by anyone. The success in this project also led us to similar transcriptome studies in another legume plant *Leucaena leucocephala*, which was successfully completed (not subject of this thesis).

APPENDIX

Summary: The purpose of this appendix is to present the entire supplementary table and figures pertaining to this research project that has not been included within the chapters.

Table S1. TSA accession numbers for genes involved in the phenylpropanoid biosynthesis pathways and plant immune response pathways in *Acacia koa*

TSA accession no.	Putative function	Hit species	% Identity	GenBank accession no.
Phenylalanine ammonia-lyase (PAL)				
GBYE01081050	PAL1	*Acacia auriculiformis x Acacia mangium*	95 %	ABD42947.1
GBYE01081051	PAL2	*Glycine max*	90 %	NP_001236956.1
Cinnamate 4-hydroxylase (C4H)				
GBYE01081054	C4H1	*Leucaena leucocephala*	95 %	AEM63594.1
GBYE01081055	C4H2	*G. max*	88 %	XP_003555891.1
4-coumarate CoA ligase (4CL)				
GBYE01081056	4CL1	*G. max*	84 %	NP_001237270.1
GBYE01081057	4CL2	*G. max*	81 %	XP_003545004.1
GBYE01081058	4CL3	*L. leucocephala*	92 %	ACI23349.1
GBYE01081059	4CL4	*L. leucocephala*	89 %	ACI23348.1
Cinnamoyl CoA reductase (CCR)				
GBYE01081068	CCR1	*L. leucocephala*	93 %	CAK22319.1
GBYE01081069	CCR3	*Cicer arietinum*	71 %	XP_004515542.1
Cinnamyl alcohol dehydrogenase (CAD)				
GBYE01081060	CAD1	*A. auriculiformis x A. mangium*	97 %	ABX75855.1
GBYE01081061	CAD2	*C. arietinum*	83 %	XP_004485621.1
4-coumarate 3-hydroxylase (C3H)				
GBYE01081062	C3H	*Caragana korshinskii*	91 %	AEV93473.1
Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT)				
GBYE01081063	HCT	*L. leucocephala*	94 %	AGA20364.1
Caffeic acid O-methyltransferase (COMT)				
GBYE01081067	COMT	*A. auriculiformis x A. mangium*	97 %	AAY86361.1
Ferulate 5-hydroxylase (F5H)				
GBYE01081070	F5H	*L. leucocephala*	89 %	ABS53040.1
Caffeoyl CoA 3-O-methyltransferase (CCoAOMT)				
GBYE01081064	CCoAOMT1	*L. leucocephala*	98 %	ABE60812.1
GBYE01081065	CCoAOMT2	*A. auriculiformis x A. mangium*	100 %	ABX75853.1
GBYE01081066	CCoAOMT3	*Musa cuminate*	73 %	XP_009413347.1
Cyclic nucleotide-gated channel (CNGC)				
GBYE01081031	CNGC5	*G. max*	81 %	XP_003519149.1
GBYE01081032	CNGC20	*G. max*	72 %	XP_003548315.1
GBYE01081033	CNGC2	*C. arietinum*	81 %	XP_004491863.1
GBYE01081034	CNGC1	*G. max*	86 %	XP_006578923.
Respiratory burst oxidase homolog (Rboh)				
GBYE01081035	Rboh C	*Medicago truncatula*	75 %	XP_003602726.1
GBYE01081036	Rboh B	*M. truncatula*	84 %	XP_003591142.1
GBYE01081037	Rboh A	*G. max*	86 %	XP_003532995.1

Table S1 (cont.). TSA accession numbers for genes involved in the phenylpropanoid biosynthesis pathways and plant immune response pathways in *Acacia koa*

TSA accession no.	Putative function	Hit species	% Identity	GenBank accession no.
Other proteins for plant immunity				
GBYE01081038	NO-associated protein 1 (NOA1)	*Cicer arietinum*	76 %	XP_004506351.1
GBYE01081039	RAR1 protein	*Glycine max*	71 %	XP_006588362.1
GBYE01081040	Chitin elicitor receptor kinase 1	*G. max*	66 %	XP_003518454.1
GBYE01081041	LysM domain-containing GPI-anchored protein 2	*Vitis vinefera*	55 %	XP_002278742.1
GBYE01081042	Pathogenesis-related protein 1	*G. max*	79 %	XP_003545770.1
GBYE01081043	WRKY29	*G. max*	44 %	XP_006580551.1

Table S2. Primer sequences used for quantitative real-time PCR (qRT-PCR) analysis

TSA accession no.	Putative function	Forward primer (5→3)	Reverse primer (5→3)
GBYE01027655	RING finger protein	CACGCAGTCTCTCACCTTGT	CATTCAACCTGCTCGGTGTG
GBYE01048118	DRE binding protein	CTTCTTCCATTCCTCTCCT	TTGAAACTGGTCGGTCTG
GBYE01041303	WRKY transcription factor	AATGCTCCTAAATCTCC	CCTCTTGTAATGGCTCGT
GBYE01019654	Zinc finger protein	GCCTTCAACTATGACCAAGAA	GATGAGACAGAGAGCGAGGT
GBYE01078745	NBS-LRR disease-resistance protein	TGGAATCTACACACCCTATCTT	GCTTTGGGAATACACCTTTC

Table S3. Microscopic analysis of cross sections of xylem in two-month-old stressed and two- and three-month-old unstressed *Acacia koa* seedlings

	2-month-old, stressed	2-month-old, unstressed	3-month-old, unstressed
Xylem cell density (cells/10^4 μm^2)	71.48 ± 2.83^a	49.07 ± 3.85^b	42.97 ± 3.49^b
Xylem cell size (μm^2)	140.43 ± 5.63^c	207.68 ± 17.74^d	235.97 ± 19.52^d

Note: Letters (a, b, c, d) indicate significant differences ($p < 0.05$) by *t*-test. Error bars represent standard error.

Table S4. Microarray analysis of sequences induced within 10-60 min following the mechanical stress treatment in *Acacia koa*

TSA acc. no.	Putative function	Fold change	SE	p-value
GBYE01027655	RING finger protein	18.07	2.99	0.047
GBYE01019260	Zinc finger protein	15.18	0.81	0.018
GBYE01019654	Zinc finger protein	14.69	3.11	0.038
GBYE01017658	Ethylene-responsive transcription factor	9.10	0.79	0.028
GBYE01026109	Protein phosphatase 2C	8.85	0.73	0.011
GBYE01048118	DRE binding protein	8.83	0.95	0.037
GBYE01041303	WRKY transcription factor	8.58	1.75	0.069
GBYE01037314	WRKY transcription factor	8.31	0.30	0.012
GBYE01039799	WRKY transcription factor	6.31	1.59	0.072
GBYE01011012	RING finger protein	6.16	0.60	0.024
GBYE01016099	WRKY transcription factor	5.56	0.64	0.040
GBYE01007796	LysM type receptor kinase	5.39	0.77	0.041
GBYE01072738	CC-NBS-IRR resistance protein	4.95	0.96	0.059
GBYE01078745	NBS-LRR disease-resistance protein	4.94	1.66	0.053
GBYE01013735	Protein phosphatase 2C	4.80	0.39	0.032
GBYE01022591	Zinc finger protein	4.66	1.08	0.006
GBYE01014926	Abscisic acid 8'-hydroxylase	4.62	0.31	0.021
GBYE01008127	Ethylene-responsive element-binding protein	4.50	0.19	0.012
GBYE01006033	Mitogen-activated protein kinase	4.25	0.43	0.008
GBYE01008850	Mitogen-activated protein kinase	4.24	0.76	0.064
GBYE01000925	Metal ion binding protein	3.74	1.22	0.022
GBYE01025591	1-aminocyclopropane 1-carboxylate synthase	3.73	0.13	0.000
GBYE01024450	NAC domain protein	3.52	0.17	0.009
GBYE01050160	NBS-LRR disease-resistance protein	3.48	0.54	0.044
GBYE01005208	Ethylene-responsive transcription factor	3.41	0.18	0.006
GBYE01003680	UDP-D-glucuronic acid 4-epimerase	3.37	0.59	0.035
GBYE01041529	Syntaxin	3.32	0.36	0.006
GBYE01050872	WRKY transcription factor	3.19	0.64	0.022
GBYE01001472	UDP-glucuronate 5-epimerase	3.14	0.37	0.003
GBYE01052680	Octicosapeptide/Phox/Bem1p (PB1)	3.12	0.77	0.031
GBYE01026684	MYB transcription factor	3.09	0.84	0.121
GBYE01025728	AP2 domain-containing transcription	3.02	0.26	0.040
GBYE01006842	Calcium-binding EF-hand protein	2.96	0.19	0.028
GBYE01014767	RING finger protein	2.79	0.30	0.026
GBYE01000421	β-1,3-glucanase	2.79	0.83	0.138
GBYE01012536	Lectin protein kinase family protein	2.78	0.39	0.006
GBYE01004300	WRKY transcription factor	2.67	0.19	0.003
GBYE01007399	Calmodulin binding protein	2.65	0.42	0.079
GBYE01000327	Chitinase	2.58	0.83	0.152
GBYE01071204	DRE binding protein	2.57	0.83	0.054
GBYE01006511	Calcium-binding protein	2.56	0.04	0.006
GBYE01005028	WRKY transcription factor	2.53	0.23	0.017
GBYE01010007	NAC domain protein	2.46	0.09	0.005
GBYE01041899	AP2/ERF domain-containing protein	2.45	0.46	0.061
GBYE01018782	Auxin-induced protein	2.44	0.62	0.097
GBYE01007979	AP2 domain class transcription factor	2.44	0.48	0.024
GBYE01019214	Protein phosphatase 2C	2.43	0.62	0.050
GBYE01007604	UDP-glucosedehydrogenase	2.29	0.44	0.033

Table S4 (cont.). Microarray analysis of sequences induced within 10-60 min following the mechanical stress treatment in *Acacia koa*

TSA accession no.	Putative function	Fold change	SE	p-value
GBYE01008935	WRKY transcription factor	2.25	0.17	0.004
GBYE01036775	AP2/ERF domain-containing protein	2.24	0.46	0.074
GBYE01030298	MYB transcription factor	2.23	0.64	0.153
GBYE01005328	Calcium-binding protein	2.23	0.42	0.021
GBYE01016418	Ser/thr protein kinase	2.22	0.12	0.016
GBYE01000487	Calmodulin	2.22	0.25	0.024
GBYE01014032	Auxin-induced protein	2.16	0.52	0.084
GBYE01013774	Cytochrome P450	2.10	0.67	0.171
GBYE01043450	Zinc finger protein	2.08	0.13	0.021
GBYE01007181	E3 ubiquitin ligase	2.07	0.35	0.019
GBYE01053827	WRKY transcription factor	2.06	0.17	0.049
GBYE01007398	Syntaxin	2.05	0.44	0.120

Table S5. Primer sequences used for Sanger sequencing

Accession no.	Putative function	Forward primer (5→3)	Reverse primer (5→3)
KX784950	Chorismate synthase (CS)	GCCCTTCAAAGTTCAATCACTC	TCCACAACTAAATACTACTACTGACC
KX784951	Chorismate mutase (CM)	TCATTCGTCTTCGCCTTCTATC	GACAATGTACTCTGCATCCATTAC
KX784949	Anthranilate synthase alpha subunit 2 (ASA2)	CCCATAGTCCACAGACACATATT	ACGAGACACGACGTCTTTATTT
KX784934	Phenylalanine ammonia-lyase (PAL)	AATTAGCTCCTCCATAATATTCCTCA	TTGCTTGAGGTCTTCACCCC
KX784943	Cinnamate 4-hydroxylase 1 (C4H)	CATAACCAATGCCACTGCAC	TTCCAACCAACCTTCATTCC
KX784935	Cinnamate 4-hydroxylase 2 (C4H)	TCTTCCCTCCCTCCCACA	CCGCGGACTAGCTAGAATCTG
KX784937	*p*-coumarate:CoA ligase (4CL)	CATCCTCATCACCAAACACG	TTGATATGGTGCGAGACACTG
KX784936	Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT)	TCTATCTTTCTCACCTTTCTGTGG	TCTGTCTCAAACGTGATCCAA
KX784938	Cinnamoyl CoA reductase (CCR)	TCCTGAGCATACCACTCCATC	AATAATAATACCCATCAGATTGCTAAC
KX784939	*p*-coumarate 3-hydroxylase (C3H)	CCTCTCTCCCACACTTCCTG	GGAGGAGACAAATGAAAACCA
KX784942	Caffeoyl-CoA O-methyltransferase (CCoAOMT)	ACTACAAGTAATGGCGGATCAA	ATTAAGCGGCATGCAATCAG
KX784941	Cinnamyl alcohol dehydrogenase (CAD)	CCGCACTTATCTCATCCTCACT	AACCAACACAGTTTCCGACA
KX784944	Caffeic acid 3-O-methyltransferase (COMT)	CGCACCAAAATGACCCTATT	CTGGCAGGCAGAATAATACAG
KX784940	Ferule 5-hydroxylase (F5H)	ATTTTAGGGGCATGCAAGT	CAAAGTTCAACCACGAAGCA
KX784933	Chalcone synthase (CHS)	GTGTTACCGGCCACTCTCAT	ACTCTGCACAGACCACAACA
KX784948	Dihydroflavonol-4-reductase (DFR)	TCCCTTATCTCCTTCCCCTTCATT	GCCAAGGCTATGGCGATGAA
KX784945	β-1,3-glucanase (Bgl)	ATCATCAACAGACATCAGAGCCA	AAGCTCCACAGCTAATAAGAGCA
KX784946	Zinc finger protein (ZFP)	AGCTCTCAATTCTCCTACTACCG	GTACAGAGAGTAAGAAGAAGGCA
KX784947	RING finger protein (RING)	CTTTCCTCCATTCTCTCTC	CCCAAGAACCTTCCTTCCTTCCT & ATTGTCACTGGTGGGTGTGA (3' RACE)

Table S6. Primer sequences used for quantitative real-time PCR (qRT-PCR) analysis

Gene name	Forward primer (5→3)	Reverse primer (5→3)	Annealing temp.
Chorismate synthase (CS)	GGTTCTTGCCTATGTCTCTCAA	TCTGTTCAAGTGTCAGAGTGTC	55 ºC
Chorismate mutase (CM)	GAACCAATGTTACCGGCTTTG	TGACTAGCCGTGGGATAAGA	55 ºC
Anthranilate synthase alpha subunit 2 (ASA2)	TGCCAGCAGGTTCAATAGG	ACTGCCTTCTTGTACTCATCAC	55 ºC
Phenylalanine ammonia-lyase (PAL)	ACGGTCACCATCAGAACAGC	GGCTTCCGATACTCCGACAC	58 ºC
Cinnamate 4-hydroxylase (C4H)	ACCAACAAGGTCGTCCAACA	TCTCACGGCCTCATTCGTTT	58 ºC
p-coumarate:CoA ligase (4CL)	GGAAGTGAAGGCAGGAGCAT	CGTGGCAAAGACAGAGAGGT	58 ºC
Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT)	CCCCTCCAACCATGAAGTCC	TGGCTTTGAGGGCATTGAGT	58 ºC
Cinnamoyl CoA reductase (CCR)	CTGCCTCCCCATCAGGTTC	TCTGGATTTCTGACGGTGCC	58 ºC
Caffeoyl-CoA O-methyltransferase (CCoAOMT)	CTGGTTACTCCCTACTCGCC	GGCCCTCTTTGAAGGTGATT	56 ºC
Chalcone synthase (CHS)	GATGAGATCAGGAAGGCTCA	GTTCGCTGTTGGTGATTCTG	57 ºC
Dihydroflavonol-4-reductase (DFR)	GTTCCGGCAGAGGATGTGAA	GTCGCCACCCCTATGGATTT	58 ºC
β-1,3-glucanase (Bgl)	ATGCTCAGATCGGTGTTTGT	TTCTTGGTTGGGATCGTAGA	56 ºC
Zinc finger protein (ZFP)	GCCTTCAACTATGACCAAGAA	GATGAGACAGAGAGCGAGGT	54 ºC
RING finger protein (RING)	CACGCAGTCTCTCACCTTGT	CATTCAACCTGCTCGGTGTG	58 ºC
Class I chitinase (Akchit1a)*	GTGCGGCCAGACTTATGATT	GGC GTC GTA GGT GTA GAA GC	58 ºC
Class III chitinase (Akchit3)*	GCATAAGTGCCGACATCAAA	AAGGGCGAGTTAGACCT	55 ºC

*primer sequences from Rushanaedy et al. (2012)

LITERATURE CITED

Abbott IA (1992) Lā‘au Hawai‘i—Traditional Hawaiian Uses of Plants. Bishop Museum Press, Honolulu, HI.

Achuo EA, Prinsen E, Hofte M (2006). Influence of drought, salt stress and abscisic acid on the resistance of tomato to *Botrytis cinerea* and *Oidium neolycopersici.* Plant Pathol 55:178-186.

Adamski DJ, Dudley NS, Morden CW, Borthakur D (2012) Genetic differentiation and diversity of *Acacia koa* populations in the Hawaiian Islands. Plant Species Biol 27:181–190.

Agrawal AA, Strauss SY, Stout MJ (1999) Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. Evolution 53:1093–1104.

Alvarez-Venegas R, Abdallat AA, Guo M, Alfano JR, Avramova Z (2007) Epigenetic control of a transcription factor at the cross section of two antagonistic pathways. Epigenetics 2:106–113.

Ambawat S, Sharma P, Yadav NR, Yadav RC (2013) MYB transcription factor genes as regulators for plant responses: An overview. Physiol Mol Biol Plants 19:307–321.

Andersen CL, Jensen JL, Ørntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250.

Anderson RC, Gardner DE (1998) Investigations of koa (*Acacia koa*) decline in Hawaiian forests. *In:* Secretariat for Conservation Biology (ed) Proceedings of the Hawaii Conservation Conference, Honolulu, HI, pp 15.

Anderson RC, Gardner DE, Daehler CC, Meinzer FC (2002) Dieback of *Acacia koa* in Hawaii: ecological and pathological characteristics of affected stands. For Ecol Manag 162:273–286.

Anten NPR, Casado-Garcia R, Nagashima H (2005) Effects of mechanical stress and plant density on mechanical characteristics, growth, and lifetime reproduction of tobacco plants. Am Nat 166:650–660.

Arteca JM, Arteca RN (1999) A multi-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase (ACS6) in mature *Arabidopsis* leaves. Plant Mol Biol 39:209–219.

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, et al. (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415:977–983.

Atkinson NJ, Urwin PE (2012) The interaction of plant biotic and abiotic stresses: from genes to the field. J Exp Bot 63:3523–3543.

Axtell MJ, Staskawicz BJ (2003) Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. Cell 112:369–377.

Ayres PG, Woolacott B (1980) Effects of soil water level on the development of adult plant resistance to powdery mildew in barley. Ann Appl Biol 94:255-263.

Baker PJ, Scowcroft PG, Ewel JJ (2009) Koa (*Acacia koa*) ecology and silviculture. General Technical Report - Pacific Southwest Research Station, USDA Forest Service.

Bakshi M, Oelmüller R (2014) WRKY transcription factors: Jack of many trades in plants. Plant Signal Behav 9:1–18.

Baldwin IT (1998). Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proceedings of the National Academy of Sciences of the USA 95: 8113–8118.

Beckers GJM, Jaskiewicz M, Liu Y, Underwood WR, He SY, et al. (2009) Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. Plant Cell 21:944–953.

Beneloujaephajri E, Costa A, L'Haridon F, Métraux JP, Binda M (2013) Production of reactive oxygen species and wound-induced resistance in *Arabidopsis thaliana* against *Botrytis cinerea* are preceded and depend on a burst of calcium. BMC Plant Biol 13:160.

Benikhlef L, L'Haridon F, Abou-Mansour E, Serrano M, Binda M, et al. (2013) Perception of soft mechanical stress in *Arabidopsis* leaves activates disease resistance. BMC Plant Biol 13:133.

Besseau S, Hoffmann L, Geoffroy P, Lapierre C, Pollet B, Legrand M (2007) Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. Plant Cell 19:148-162.

Beyl CA, Mitchell CA (1983) Alteration of growth, exudation rate, and endogenous hormone profiles in mechanically dwarfed sunflower. J Amer Soc Hort Sci 108:257-262.

Biddington NL, Dearman AS (1985) The effect of mechanically induced stress on the growth of cauliflower, lettuce and celery seedlings. Ann Bot 55:109–119.

Bilgin DD, DeLucia EH, Clough SJ (2009) A robust plant RNA isolation method suitable for Affymetrix GeneChip analysis and quantitative real-time RT-PCR. Nat Protoc 4:333340.

Biro R, Hunt ER, Erner Y, Jaffe MJ (1980) Thigmomorphogenesis: changes in cell division and elongation in the internodes of mechanically-perturbed or ethrel-treated bean plants. Ann Bot 45:655-664.

Biro R, Jaffe M (1984) Thigmomorphogenesis: ethylene evolution and its role in the changes observed in mechanically perturbed bean plants. Physiol Plant 62:289–296.

Bögre L, Ligterink W, Meskiene I, Barker P, Heberle-Bors E, et al. (1997) Wounding Induces the Rapid and Transient Activation of a Specific MAP Kinase Pathway. Plant Cell 9:75–83.

Bostock RM (2005) Signal crosstalk and induced resistance: straddling the line between cost and benefit. Annu Rev Phytopathol 43:545–580.

Botella JR, Arteca JM, Somodevilla M, Arteca RN (1996) Calcium-dependent protein kinase gene expression in response to physical and chemical stimuli in mungbean (*Vigna radiata*) Plant Mol Biol 30:1129–1137.

Botella JR, Arteca RN (1994) Differential expression of two calmodulin genes in response to physical and chemical stimuli. Plant Mol Biol 24:757–766.

Botella JR, Arteca RN, Frangos JA (1995) A mechanical strain-induced 1-aminocyclopropane-1-carboxylic acid synthase gene. Proc Natl Acad Sci USA 92:1595–1598.

Boubakri H, Poutaraud A, Wahab MA, Clayeux C, Baltenweck-Guyot R, et al. (2013) Thiamine modulates metabolism of the phenylpropanoid pathway leading to enhanced resistance to *Plasmopara viticola* in grapevine. BMC Plant Biol 13:31.

Bourgeade P, Boyer N, de Jaegher G, Gaspar T (1989) Carry-over of thigmomorphogenetic characteristics in calli derived from *Bryonia dioicainternodes*. Plant Cell Tissue Organ Cult 19:199–211.

Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X (1997) The cpr5 mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell 9:1573–1584.

Bowling SA, Guo A, Cao H, Gordon AS, Klessig D, Dong X (1994) A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. Plant Cell 6:1845–1857.

Boyes DC, Nam J, Dangl JL (1998) The *Arabidopsis thaliana* RPM1 disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. Proc Natl Acad Sci USA 95:15849–15854.

Braam J (2005) In touch: plant responses to mechanical stimuli. New Phytol 165:373–89.

Braam J, Davis RW (1990) Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. Cell 60:357–364.

Brewbaker JL (1997) Genetic improvement, a *sine qua non* for the future of koa. *In:* Ferentinos L, Evans DO (eds) Koa: a decade of growth. Proceedings of the symposium, Hawaii Forest Industry Association, Honolulu, HI, pp 24-26.

Brewbaker JL, Sorensson CT, Wheeler RW (1990) New tree crops from interspecific *Leucaena* hybrids *In:* Janick J, Simon JE (eds) Advances in new crops. Timber Press, Portland. pp 283-289.

Broekaert WF, Terras FRG, Cammue BPA (2000) Induced and preformed antimicrobial proteins. *In*: Slusarenko AJ, Fraser RSS, Van Loon LC (eds). Mechanisms of Resistance to Plant Diseases. Dordrecht, The Netherlands: Kluwer Academic Publishers. pp 371-478.

Brutus A, Sicilia F, Macone A, Cervone F, Lorenzo GD (2010) A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. Proc Natl Acad Sci USA 107 9452–9457

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113-116.

Chassot C, Buchala A, Schoonbeek HJ, Me´traux JP, Lamote O. (2008) Wounding of *Arabidopsis* leaves causes a powerful but transient protection against *Botrytis* infection. Plant Journal 55: 555–567.

Chehab EW, Eich E and Braam J (2009) Thigmomorphogenesis: A complex plant response to mechano-stimulation. J Exp Bot, 43–56.

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, et al. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature 448:497–500.

Choudhary DK, Prakash A, Johri BN (2007) Induced systemic resistance (ISR) in plants: mechanism of action. Indian J Microbiol 47:289-297.

Cicco N, Lanorte MT, Paraggio M, Viggiano M, Lattanzio V (2009) A reproducible, rapid and inexpensive Folin-Ciocalteu micro-method in determining phenolics of plant methanol extracts. Microchem J 91:107-110.

Cipollini DF (2002) Does competition magnify the fitness costs of induced responses in *Arabidopsis thaliana*? A manipulative approach. Oecologia 131:514–520.

Cipollini DF Jr (1997) Wind-induced mechanical stimulation increases pest resistance in common bean. Oecologia 111:84-90.

Clough SJ, Fengler KA, Yu IC, Lippok B, Smith RK, et al. (2000) The *Arabidopsis* dnd1 "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. Proc Natl Acad Sci USA 97:9323–9328.

Cohen YR (2002) β-aminobutyric acid-induced resistance against plant pathogens. Plant Dis 86:448-457.

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, et al. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674–3676.

Conrath U (2006) Systemic acquired resistance. Plant Sig Behav 1(4):179-184.

Copois V, Bibeau F, Bascoul-Mollevi C, Salvetat N, Chalbos P, et al. (2007) Impact of RNA degradation on gene expression profiles: assessment of different methods to reliably determine RNA quality. J Biotechnol 127(4):549-559.

Coutand C, Julien JL, Moulia B, Mauget JC, Guitard D (2000) Biomechanical study of the effect of a controlled bending on tomato stem elongation: global mechanical analysis. J Exp Bot 51:1813–1824.

Daehler CC, Yorkston M, Sun W, Dudley N (1999) Genetic variation in morphology and growth characters of *Acacia koa* in the Hawaiian Islands. Int J Plant Sci 160:767–773.

de Jaegher G, Boyer N, Bon MC, Gaspar T (1987) Thigmomorphogenesis in *Bryonia dioica*: early events in ethylene biosynthesis pathway. Biochem Physiol Pflanz 182:49–56.

de Jaegher G, Boyer N, Gaspar T (1985) Thigmomorphogenesis in *Bryonia dioica*: changes in soluble and wall peroxidases, phenylalanine ammonia-lyase activity, cellulose, lignin content and monomeric constituents. Plant Growth Regul 3:133-148.

Decreux A, Thomas A, Spies B, Brasseur R, Cutsem PV, Messiaen J (2006) In vitro characterization of the homogalacturonan-binding domain of the wall-associated kinase WAK1 using site-directed mutagenesis. Phytochem 67:1068–1079

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, et al. (1994) A central role of salicylic acid in plant disease resistance. Science 266:1247–1250.

Delessert C, Wilson IW, Van Der SD, Dennis ES, Dolferus R (2004) Spatial and temporal analysis of the local response to wounding in *Arabidopsis* leaves. Plant Mol Biol 55:165–181.

Diourte M, Starr JL, Jeger MJ, Stack JP, Rosenow DT (1995) Charcoal rot (*Macrophomina phaseolina*) resistance and the effects of water-stress on disease development in sorghum. Plant Pathol 44:196-202.

Duan J, Xia C, Zhao G, Jia J, Kong X (2012) Optimizing *de novo* common wheat transcriptome assembly using short-read RNA-Seq data. BMC Genomics 13:392.

Duan L, Liu H, Li X, Xiao J, Wang S (2014) Multiple phytohormones and phytoalexins are involved in disease resistance to *Magnaporthe oryzae* invaded from roots in rice. Physiologia Plantarum 152:486–500.

Dudley N (2007) *Acacia* utilisation and management: adding value. *In:* Beadle CL, Brown AG (eds) RIRDC Publication No. 07/095, Rural industries research and development corporation, Canberra.

Dudley N, James R, Sniezko R, Cannon P, Yeh A, et al. (2008) Operational disease screening program for resistance to wilt in *Acacia koa* in Hawaii. *In:* PSW-GTR-240. U.S. Department of Agriculture FS, Pacific Southwest Research Station, Eugene, OR.

Dudley N, Yamasaki J (2000) A guide to determining wood properties of *Acacia koa*. HARC Forestry Report No. 3. http://www.hawaiiag.org/harc. Accessed 20 June (2016)

Dudley NS, James RL, Sniezko RA, Yeh A (2007*a*) Investigating koa wilt and dieback in Hawaii: pathogenicity of *Fusarium* species on *Acacia koa* seedlings. Native Plants J 8(3):259-266.

Dudley NS, James RL, Yeh A (2007*b*) Comparative virulence of Hawaiian *Fusarium oxysporum* isolates on *Acacia koa* seedlings. Forest health Protection. United States Department of Agriculture, Forest Service, Northern Region, Missoula, MT.

Dudley NS, Jones TC, James RL, Sniezko RA, Cannon P, Borthakur D (2015) Applied disease screening and selection program for resistance to vascular wilt in Hawaiian *Acacia koa*. South For 77:65–73.

Durrenberger PF, Fernando S, Kashefi SN, Ferrer I, Hauw JJ, et al. (2010) Effects of antemortem and postmortem variables on human brain mRNA quality: a BrainNet Europe study. J Neuropathol Exp Neurol 69:70-81.

Ehlting J, Shin JJ, Douglas CJ (2001) Identification of 4-coumarate:coenzyme A ligase (4CL) substrate recognition domains. Plant J 27:455-465.

Elevitch CR, Wilkinson KM, Friday JB (2006) *Acacia koa* (koa) and *Acacia koaia* (koai'a). *In:* Elevitch CR (ed) Species profiles for Pacific Island agroforestry, Hōlualoa.

Elsharkawya MM, Hase T, Yagi Y, Shimizu M, Hyakumachi M (2015) Induction of systemic resistance against Fusarium crown and root rot disease by blast processing. J Plant Interact 10(1):262-269.

Erner Y, Jaffe MJ (1982) Thigmomorphogenesis: the involvement of auxin and abscisic acid in growth retardation due to mechanical perturbation. Plant Cell Physiol 23:935–941.

Escoll P, Mondino S, Rolando M, Buchrieser C (2016) Targeting of host organelles by pathogenic bacteria:a sophisticated subversion strategy. Nat Rev Microbiol 14:5-19.

Ewel JJ, Bigelow SW (1996) Plant life forms and tropical ecosystem functioning. *In*: Orians GH, Dirzo R, Cushman JH (eds) Biodiversity and Ecosystem Processes in Tropical Forests. Springer-Verlae, pp 101-126.

Fleige S, Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med 27:126-139.

Fredua-Agyeman R, Adamski D, Liao RJ Morden C, Borthakur D (2008) Development and characterization of microsatellite markers for analysis of population differentiation in the tree legume *Acacia koa* (Fabaceae: Mimosoideae) in the Hawaiian Islands. Genome. 51(12):1001–1015.

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, et al. (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261:754–756.

Gambino G, Perrone I, Gribaudo I (2008) A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. Phytohchem Anal 19: 520-525.

Gardner DE (1980) *Acacia koa* seedling wilt caused by *Fusarium oxysporum* f. sp. *koae*, f. sp. *nov*. Phytopathology 70: 594–597.

Gardner DE (1996) *Acacia koa:* A review of its diseases and associated fungi. *In:* Ferentinos L, Evans DO (eds) Koa: a decade of growth. Proceedings of the symposium, Hawaii Forest Industry Association, Honolulu, HI, pp 56–63.

Garg R, Patel RK, Tyagi AK, Jain M (2011) *De novo* assembly of chickpea transcriptome using short reads for gene discovery and marker identification. DNA Res 18: 53–63.

Goellner K, Conrath U (2008) Priming: it's all the world to induced disease resistance. Eur J Plant Pathol 121:233-242.

Gómez-Gómez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol Cel 5(6):1003-11.

Good NE, Andreae W (1957) Malonyltryptophan in higher plants. Plant Physiol 32: 561–566.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29:644–652.

Gruenheit N, Deusch O, Esser C, Becker M, Voelckel C, et al. (2012) Cutoffs and k-mers: implications from a transcriptome study in allopolyploid plants. BMC Genomics 13: 92.

Guzmán P (2012) The prolific ATL family of RING-H2 ubiquitin ligases. Plant Signal Behav 7:1014–1021.

Hamel F, Boivin R, Tremblay C, Bellemare G (1997) Structural and evolutionary relationships among chitinases of flowering plants. J Mol Evol 44:614–624.

Hamilton JP, Buell CR (2012) Advances in plant genome sequencing. Plant J 70:177–190.

Hammond-Kosack KE, Kanyuka K (2007) Resistance genes (R genes) in plants. Encycl Life Sci 40:1-21.

Harrington RA, Fownes JH, Meinzer FC, Scowcroft PG (1995) Forest growth along a rainfall gradient in Hawaii: *Acacia koa* stand structure, productivity, foliar nutrients, and water- and nutrient-use efficiencies. Oecologia 102:277–284.

Heidel AJ, Clarke JD, Antonovics J, Dong X (2004) Fitness costs of mutations affecting the systemic acquired resistance pathway in *Arabidopsis thaliana*. Genetics 168: 2197–2206.

Heil M, Hilpert A, Kaiser W, Linsenmair KE (2000) Reduced growth and seed set following chemical induction of pathogen defence: Does systemic acquired resistance (SAR) incur allocation costs? J Ecol 88: 645–654.

Hepworth D, Vincent J (1999) The growth response of the stems of genetically modified tobacco plants (*Nicotiana tabacum* "Samsun") to flexural stimulation. Ann Bot 83:39–43.

Hewezi T, Léger M, Gentzbittel L (2008) A comprehensive analysis of the combined effects of high light and high temperature stresses on gene expression in sunflower. Ann Bot 102(1):127-140.

Hirayama M, Wada Y, Nemoto H (2006) Estimation of drought tolerance based on leaf temperature in upland rice breeding. Breed Sci 5647-54.

Howe GA (2004) Jasmonates as signals in the wound response. J. Plant Growth Regul. 23:223–237.

Huffaker A, Ryan CA (2007) Endogenous peptide defense signals in *Arabidopsis* differentially amplify signaling for the innate immune response. Proc Natl Acad Sci USA 104:10732–10736.

Ishihara K, Lee EKW, Borthakur D (2016) An improved method for RNA extraction from woody legume species *Acacia koa A*. Gray and *Leucaena leucocephala* (Lam.) de Wit. *Int*. J Forestry Wood Sci 3:31-35.

Ishihara K, Lee EKW, Rushanaedy I, Borthakur D (2015) Illumina-based de novo transcriptome analysis and identifications of genes Involved in the monolignol biosynthesis pathway in *Acacia koa*. Am J Bioinforma 4(1):7–27.

Jacobs MR (1954) The effect of wind sway on the form and development of *Pinus radiata*. Austral J Bot 2:35-51.

Jaffe MJ (1973) Thigmomorphogenesis: The response of plant growth and development to mechanical stimulation. Planta 114:143–157.

Jaffe MJ, Biro R (1979) Thigmomorphogenesis: the effect of mechanical perturbation on the growth of plants, with special reference to anatomical changes, the role of ethylene, and interaction with other environmental stresses. In Mussell H, Staples RC, eds, Stress Physiology in Crop Plants. John Wiley & Sons, New York, pp 25–69.

James RL (2005) Pathogenic comparison of *Fusarium* isolates from diseased Hawaiian *Acacia koa*. Nursery Disease Notes No. 164, USDA Forest Service Northern Region.

Janse JD, Obradovic A (2010) *Xylella fastidiosa*: its biology, diagnosis, control and risks. J Plant Pathol 92(1):S1.35-S1.48.

Jaskiewicz M, Conrath U, Peterhänsel C (2011) Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. EMBO Rep 12(1):50-5.

Jayashree B, Punna R, Prasad P, Bantte K, Hash CT, et al. (2006) A database of simple sequence repeats from cereal and legume expressed sequence tags mined in silico: survey and evaluation. In Silico Biol 6(6):607–620.

Jenns AE, Kuc J (1979) Graft transmission of systemic resistance of cucumber to anthracnose induced by *Colletotrichum lagenarium* and tobacco necrosis virus. Phytopathology 7:753–756.

Johnson KA, Sistrunk ML, Polisensky DH and Braam J (1998) *Arabidopsis thaliana* responses to mechanical stimulation do not require ETR1 or EIN2. Plant Physiol 116:643–9.

Johnson MTJ, Carpenter EJ, Tian Z, Bruskiewich R, Burris JN et al. (2012) Evaluating methods for isolating total RNA and predicting the success of sequencing phylogenetically diverse plant transcriptomes. PLoS ONE 7(11):e50226.

Jordon-Thaden I, Chanderbali A, Gitzendanner M, Soltis D (2015) Modified CTAB and TRIzol protocols improve RNA extraction from chemically complex *Embryophyta*. Appl Plant Sci 3(5):1400105-1400105.

Joshi CP, Chiang VL (1998) Conserved sequence motifs in plant S-adenosyl-L-methionine-dependent methyltransferases. Plant Molecular Biol 37: 663-674.

Kamphuis LG, Williams AH, Küster H, Trengove RD, Singh KB, et al. (2012) *Phoma medicaginis* stimulates the induction of the octadecanoid and phenylpropanoid pathways in *Medicago truncatula*. Mol Plant Pathol 13:593–603.

Kasprzewska A (2003) Plant chitinases - regulation and function. Cell Mol Biol Lett 8:809–824.

Kato H, Asai S, Yamamoto-Katou A, Yoshioka H, Doke N, et al. (2008) Involvement of NbNOA1 in NO production and defense responses in INF1-treated *Nicotiana benthamiana*. J Gen Plant Pathol 74:15–23.

Kiba A, Maimbo M, Kanda A, Tomiyama H, Ohnishi K, Hikichi Y (2007) Isolation and expression analysis of candidate genes related to *Ralstonia solanacearum*-tobacco interaction. Plant Biotechnol 24:409–416.

Kim JM, To TK, Ishida J, Matsui A, Kimura H, et al. (2012) Transition of chromatin status during the process of recovery from drought stress in *Arabidopsis thaliana*.53(5):847-56.

Kim JM, To TK, Ishida J, Morosawa T, Kawashima M, et al. (2008) Alterations of lysine modifications on the histone H3 N-tail under drought stress conditions in *Arabidopsis thaliana*. Plant Cell Physiol 49(10):1580-8.

Kishimoto K, Kouzai Y, Kaku H, Shibuya N, Minami E, et al. (2010) Perception of the chitin oligosaccharides contributes to disease resistance to blast fungus *Magnaporthe oryzae* in rice. Plant J 64:343–354.

Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, et al. (2007) Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell 19:1065–1080.

Kohler A, Schwindling S, Conrath U. (2002) Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the NPR1/NIM1 gene in *Arabidopsis*. Plant Phys 128:1046-1056.

Kohorn BD, Kohorn SL (2012) The cell wall-associated kinases, WAKs, as pectin receptors. Front Plant Sci 3:88.

Koller D (2011) The restless plant. Volkenburgh EV, ed. Massachusetts: Harvard University Press.

Komada H (1975) Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Rev Plant Protec Res 8:114–125.

Krauss BH (1993) Plants in Hawaiian Culture. University of Hawai'i Press, Honolulu, HI.

Kuc J, Richmond S (1977) Aspects of the protection of cucumber against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. Phytopathology 67:533–536.

Kumpatla SP, Mukhopadhyay S (2005) Mining and survey of simple sequence repeats in expressed sequence tags of dicotyledonous species. Genome 48(6): 85–998.

Larsen K (2004) Molecular cloning and characterization of cDNAs encoding cinnamoyl CoA reductase (CCR) from barley (*Hordeum vulgare*) and potato (*Solanum tuberosum*). J Plant Physiol 161:105-112.

Lata C, Prasad M (2011) Role of DREBs in regulation of abiotic stress responses in plants. J Exp Bot 62:4731–4748.

Lee D, Polisensky DH, Braam J (2005) Genome-wide identification of touch- and darkness-regulated *Arabidopsis* genes: A focus on calmodulin-like and XTH genes. New Phytol 165:429–444.

Lev-Yadun S, Flaishman MA (2001) The effect of submergence on ontogeny of cambium and secondary xylem and on fiber lignification in inflorescence stems of *Arabidopsis.* IAWA J 22:159–169.

Li J, Brader G, Palva ET (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell 16(2):319-331.

Liu B, Li JF, Ao Y, Qu J, Li Z, et al. (2012) Lysin motif-containing proteins LYP4 and LYP6 play dual roles in peptidoglycan and chitin perception in rice innate immunity. Plant Cell 24:3406–3419

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408.

Loomis WD (1974). Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods Enzymol 31:528-544.

Loudat TA, Kanter R (1996) The Economics of Commercial Koa Culture in Hawaii. *In:* Ferentinos L, Evans DO (eds) Koa: a decade of growth. Proceedings of the symposium, Hawaii Forest Industry Association, Honolulu, HI, pp 124–147.

Lu S, Friesen TL, Faris JD (2011) Molecular characterization and genomic mapping of the pathogenesis-related protein 1 (PR-1) gene family in hexaploid wheat (*Triticum aestivum L.*) Mol Genet Genomics 285:485–503.

Luna E, Bruce TJA, Roberts MR, Flors V, Ton J (2012) Next-generation systemic acquired resistance. Ame Soc of Plant Biol.158(2):844-853.

Luna E, Ton J (2012) The epigenetic machinery controlling transgenerational systemic acquired resistance Plant Sig Behav 7(6):615-618.

Luo M, Liang XQ, Dang P, Holbrook CC, Bausher MG, et al. (2005) Microarray-based screening of differentially expressed genes in peanut in response to *Aspergillus parasiticus* infection and drought stress. Plant Sci 169(4):695-703.

Lynch D, Lidgett A, McInnes R, Huxley H, Jones E, et al. (2002) Isolation and characterization of three cinnamyl alcohol dehydrogenase homologue cDNAs from perennial ryegrass (*Lolium perenne* L.). J Plant Physiol 159:653-660.

Ma W, Smigel A, Tsai YC, Braam J, Berkowitz GA (2008) Innate immunity signaling:cytosolic Ca2+ elevation is linked to downstream nitric oxide generation through the action of calmodulin or a calmodulin-like protein. Plant Physiol 148:818–828.

MacHardy WE, Beckman CH (1981) Vascular wilt *Fusaria*: infections and pathogenesis. *In*: Nelson PE, Toussoun TA and Cook RJ (eds) *Fusarium*: Diseases, Biology and Taxonomy. The Pennysylvania State University Press, University Park, London, pp 365-390.

Marino D, Dunand C, Puppo A, Pauly N (2012) A burst of plant NADPH oxidases. Trends Plant Sci 17:9–15.

Mayek-Perez N, Garcia-Espinosa R, Lopez-Castaneda C, Acosta-Gallegos JA, Simpson J (2002) Water relations, histopathology and growth of common bean *(Phaseolus vulgaris L*.) during pathogenesis of *Macrophomina phaseolina* under drought stress. Physiol Mol Plant Pathol 60:185-195.

Mayer MJ, Narbad A, Parr AJ, Parker ML, Walton NJ, et al. (2001) Rerouting the plant phenylpropanoid pathway by expression of a novel bacterial enoyl-CoA hydratase/lyase enzyme function. Plant Cell 13:1669–1682.

McHale L, Tan X, Koehl P, Michelmore RW (2006) Plant NBS-LRR proteins: adaptable guards. Genome Biol 7:212.

McKie JH, Jaouhari R, Douglas KT, Goffner D, Feuillet C, et al. (1993) A molecular model for cinnamyl alcohol dehydrogenase, a plant aromatic alcohol dehydrogenase involved in lignification. Biochim Biophys Acta 1202:61-69.

Miles MR, Wilcoxson RD (1984) Production of fungal inoculum using a substrate of perlite, cornmeal, and potato-dextrose agar. Plant Dis 68:310.

Mishra PK, Fox RTV, Culham A (2003) Development of a PCR-based assay for rapid and reliable identification of pathogenic *Fusaria*. FEMS Microbiol Lett 218: 329–332.

Mitchell C, Ogura C, Meadows DW, Kane A, Strommer L, Fretz S, Leonard D, McClung A (2005) Hawaii's Comprehensive Wildlife Conservation Strategy. Department of Land and Natural Resources, Honolulu, HI.

Mitchell CA, Severson CJ, Wott JA, Hammer PA (1975) Seismomorphogenic regulation of plant growth. J Amer Soc Hortic Sci 100:161–165.

Mittler R, Kim Y, Song L, Coutu J, Coutu A, Ciftci-Yilmaz S, Lee H, Stevenson B, Zhu JK (2006) Gain- and loss-of-function mutations in Zat10 enhance the tolerance of plants to abiotic stress. FEBS Lett 580:6537–6542.

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, et al. (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. Proc Natl Acad Sci USA 104:19613–19618.

Mizoguchi T, Irie K, Hirayama T, Hayashida N, Yamaguchi-Shinozaki K, et al. (1996) A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 93:765-9.

Moreau M, Gyu IL, Wang Y, Crane BR, Klessig DF (2008) AtNOS/AtNOA1 is a functional *Arabidopsis thaliana* cGTPase and not a nitric-oxide synthase. J Biol Chem 283:32957–32967.

Mueller-Dombois D, Fosberg FR (1998) *Acacia koa* forest. *In*: Vegetation of the tropical Pacific islands. Springer, NY, pp 506–511.

Muskett PR, Kahn K, Austin MJ, Moisan LJ, Sadanandom A, et al. (2002) *Arabidopsis* RAR1 exerts rate-limiting control of R gene-mediated defenses against multiple pathogens. Plant Cell 14:979–992.

Nakasugi K, Crowhurst R, Bally J, Waterhouse P (2014) Combining transcriptome assemblies from multiple de novo assemblers in the allo-tetraploid plant *Nicotiana benthamiana*. PLoS One 9:e91776.

Nassuth A, Pollari E, Helmeczy K, Stewart S, Kofalvi SA (2000) Improved RNA extraction and one-tube RT-PCR assay for simultaneous detection of control plant RNA plus several viruses in plant extracts. J Virol Methods 90(1):37-49.

Negi VS, Pal A, Singh R, Borthakur D (2011) Identification of species-specific genes from *Leucaena leucocephala* using interspecies suppression subtractive hybridisation. Ann Appl Biol 159:387–398.

Newell LA, Buck M (1996) Hawaii forestry: opportunities and uncertainties. J Forest 94(10):4–8.

Nuruzzaman M, Sharoni AM, Kikuchi S (2013) Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. Front Microbiol 4:248.

O'Donnell K, Kistler HC, Cigelnik E & Ploetz RC (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proc Natl Acad Sci USA 95:2044–2049.

Oerke EC, Krone C, Jacobi I, Schonbeck F (1992) Relations between stress-induced modifications of the pathogenesis of *Erysiphe graminis hordei* and the membrane components of barley. J Phytopathol 134:157-169.

Oerke EC, Schonbeck F (1986) On the influence of abiotic stress conditions on growth of barley and bean and their predoposition for pathogens. J Plant Dis Prot 93:561-573.

Oh SA, Kwak JM, Kwun IC, Nam HG (1996) Rapid and transient induction of calmodulin-encoding gene(s) of Brassica napus by a touch stimulus. Plant Cell Rep 15:586–590.

Paul-Victor C, Rowe N (2011) Effect of mechanical perturbation on the biomechanics, primary growth and secondary tissue development of inflorescence stems of *Arabidopsis thaliana*. Ann Bot 107:209–218.

Pejchar L, Press D (2006) Achieving conservation objectives through production forestry: the case of *Acacia koa* on Hawaii Island. Environl Sci Policy 9(5):439-447.

Perkins KS, Nimmo JR, Medeiros AC, Szutu DJ, von Allmen E (2014) Assessing effects of native forest restoration on soil moisture dynamics and potential aquifer recharge, Auwahi, Maui. Ecohydrology 7(5):1437–1451.

Pieterse CM, Zamioudis C, Berendsen RL, Weller DM, Van Wees SC, Bakker PA (2014) Induced systemic resistance by beneficial microbes.. Annu Rev Phytopathol 52:347-75.

Pietro A, Madrid MP, Caracuel Z, Delgado-Jarana J, Roncero MIG (2003) *Fusarium oxysporum:* Exploring the molecular arsenal of a vascular wilt fungus. Mol Plant Pathol 4:315–325.

Plomion C, Leprovost G, Stokes A (2001) Wood formation in trees. Plant Physiology. 127(4):1513–1523.

Porra R (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls *a* and *b*. Photosynth Res 73:149–156.

Porter BW, Zhu YJ, Webb DT, Christopher D (2009) Novel thigmomorphogenetic responses in *Carica papaya*: Touch decreases anthocyanin levels and stimulates petiole cork outgrowths. Ann Bot 103:847–858.

Presti LL, Lanver D, Schweizer G, Tanaka S, Liang L, et al. (2015) Fungal effectors and plant susceptibility. Annu Rev Plant Viol 66:513-45.

Pruyn ML, Ewers III BJ, Telewski FW (2000) Thigmomorphogenesis: changes in the morphology and mechanical properties of two *Populus* hybrids in response to mechanical perturbation. Tree Physiol 20:535–540.

Punja ZK, Zhang YY (1993) Plant chitinases and their roles in resistance to fungal diseases. J Nermatol 25(4):526-540.

Purwar S (2012) Enzymes of phenylpropanoid metabolism involved in strengthening the structural barrier for providing genotype and stage dependent resistance to Karnal Bunt in wheat. Am J Plant Sci 3: 261–267.

Qi D, DeYoung BJ, Innes RW (2012) Structure-function analysis of the coiled-coil and leucine-rich repeat domains of the RPS5 disease resistance protein. Plant Physiol 158:1819–1832.

Rainey PB (1999) Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. Environ Microbiol 1(3):243-57.

Ramegowda V, Senthil-Kumar M, Ishiga Y, Kaundal A, Udayakumar M, et al. (2013) Drought stress acclimation impacts tolerance to *Sclerotina sclerotiorum* and *Pseudomonas syringae* in *Nicotiana benthamiana.* Int J Mol Sci 14:9497–9513.

Rekoslavskaya N, Shvetsov SG, Markova T, Gamburg KZ (1992) Induction of N-malonyl-D-tryptophan by drought stress: Is D-tryptophan the only D-amino acid appeared in wilted leaves? Biol Plant 34(3–4):297–304.

Rekoslavskaya NI (1986) Possible role of N-malonyl-D-tryptophan as an auxin precursor. Biologia Plantarum 28:62.

Remers WA (1972) Properties and reactions of indoles, isoindoles, and their hydrogenated derivatives. *In*: Houlihan WJ (ed) Indoles part one. Wiley Interscience, London. pp 1–226.

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell 12:707–720.

Rispail N, Rubiales D (2015) Rapid and efficient estimation of pea resistance to the soil-borne pathogen *Fusarium oxysporum* by infrared imaging. Sensors (Basel) 15(2):3988-4000.

Rizhsky L, Liang HJ, Mittler R (2002) The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiol 130:1143-1151.

Rizhsky L, Liang HJ, Shuman J, Shulaev V, Davletova S, Mittler R (2004) When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. Plant Physiol 134:1683-1696.

Rodriguez-Cruz SE (2005) Analysis and Characterization of Designer Tryptamines using Electrospray Ionization Mass Spectrometry (ESI-MS) *In*: ASMS 17th Sanibel Conference on Mass Spectrometry - Mass Spectrometry in Forensic Science and Counterterrorism, Clearwater Beach, FL.

Romero AM, Kousik CS, Ritchie DF. (2001) Resistance to bacterial spot in bell pepper induced by acibenzolar-S-methyl. Plant Dis 85:189–194.

Ross AF (1961) Localized acquired resistance to plant virus infection in hypersensitive hosts. Virology 14:329–339.

Rossel JB, Wilson PB, Hussain D, Woo NS, Gordon MJ, Mewett OP, Howell J, Whelan J, Kazan K, Pogson BJ (2007) Systemic and intracellular responses to photooxidative stress in *Arabidopsis*. Plant Cell 19:4091–4110.

Rushanaedy I, Jones TC, Dudley NS, Liao RJF, Agbayani R, Borthakur D (2012) Chitinase is a potential molecular biomarker for detecting resistance to *Fusarium oxysporum* in *Acacia koa*. Trop Plant Biol 5:244–252.

Sahu J, Sen P, Choudhury MD, Barooah M, Modi MK, et al. (2012) Towards an efficient computational mining approach to identify EST-SSR markers. Bioinformation 8(4):201-202.

Saidi I, Ammar S, Demont-Caulet N, Thévenin J, Lapierre C, Bouzie S, Jouanin L (2009) Thigmomorphogenesis in *Solanum lycopersicum*: Morphological and biochemical responses in stem after mechanical stimulation. Plant Sci 177:1–6.

Sakai HF (1988) Avian response to mechanical clearing of a native rainforest in Hawaii. Condor 90:339-348.

Savatin DV, Giovanna G, Modesti V, Gervone F (2014) Wounding in the plant tissue:the defense of a dangerous passage. Front Plant Sci 5:470.

Schuler MA (1996) Plant cytochrome P450 monooxygenases. Critical Rev Plant Sci 15:235-284.

Scowcroft PG, Friday JB, Haraguchi J, Idol T, Dudley NS (2010) Poor stem form as a potential limitation to private investment in koa plantation forestry in Hawaii. Small-scale Forestry 9: 243-262.

Seo S, Okamoto M, Seto H, Ishizuka K, Sano H, Oha Y (1995) Tobacco MAP kinase: a possible mediator in wound signal transduction pathways. Science 270:1988–1992.

Shadle GL, Wesley SV, Korth KL, Chen F, Lamb C, Dixon RA (2003) Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of L-phenylalanine ammonia-lyase. Phytochemistry 64: 153–161.

Shaik R, Ramakrishna W (2013) Genes and co-expression modules common to drought and bacterial stress responses in Arabidopsis and rice. PLoS One 8(10):e77261.

Sharma N, Sharma KP, Gaur RK, Gupta VK (2011) Role of chitinase in plant defense. Asian J Biochem 6(1):29-37.

Sharma RC, Duveiller E, Ortiz-Ferrara G (2007) Progress and challenge towards reducing wheat spot blotch threat in the Eastern Gangetic Plains of South Asia: is climate change already taking its toll? Field Crops Research 103:109-118.

Shawish O, Baker R (1982) Thigmomorphogenesis and predisposition of hosts to Fusarium wilt.Phytopathology 72:63–68.

Shi X (2003) Genetic improvement of Leucaena spp. and *Acacia koa* Gray as high-value hardwoods. Dissertation, University of Hawai'i.

Shi X, Brewbaker JL (2004) Genetic improvement of Hawaii's premier hardwood*, Acacia koa*. For Genet Resour 31:36–40.

Sims DA, Gamon JA (2002) Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. Remote Sens Environ 81:337–354.

Skolmen RG, Horiuchi H, Goo D, Masaki C (1991) Field trials, plantations, and arboreta. *In:* Brewbaker JL, Glover N, Moore E (eds) Improvement of Acacia koa: resource documents. Proceedings of workshop, April 18–20, 1991. Kamuela, HI, pp 19–21.

Slaughter A, Daniel X, Flors V, Luna E, Hohn B, et al. (2012) Descendants of primed *Arabidopsis* plants exhibit enhanced resistance to biotic stress. Plant Physiol 158:835–843

Smith VC, Ennos AR (2003) The effects of air flow and stem flexure on the mechanical and hydraulic properties of the stems of sunflowers *Helianthus annuus L*. J Exp Bot 54:845–849.

Song QJ, Shi JR, Singh S, Fickus EW, Costa JM, et al. (2005) Development and mapping of microsatellite (SSR) markers in wheat. Theor Appl Genet 110(3): 550–560.

Stahl E, Bellwon P, Huber S, Schlaeppi K, Bernsdorff F, et al. (2016) Regulatory and functional aspects of indolic metabolism in plant systemic acquired resistance. Mol Plant 9(5):662-81.

Sun W (1996) Genetic improvement of *Leucaena* and *Acacia koa*. Dissertation, University of Hawaii.

Sun W, Brewbaker JL, Austin MT (1997) Genetic variations of *Acacia koa* seed, seedling, and early growth traits. *In:* Ferentinos L, Evans DO (eds) Koa: a decade of growth. Proceedings of the symposium, Hawaii Forest Industry Association, Honolulu, HI, pp 33–37.

Sun Y, Li L, Macho A. P, Han Z, Hu Z, Zipfel C, et al. (2013) Structural basis for flg22-induced activation of the *Arabidopsis* FLS2-BAK1 immune complex. Science 342:624–628.

Swindell WR (2006) The association among gene expression responses to nine abiotic stress treatments in *Arabidopsis thaliana*. Genetics 174:1811-1824.

Taguchi Y, Elsharkawy MM, Hassan NMS, Hyakumachi M (2014) A novel method for controlling rice blast disease using fan-forced wind on paddy fields. Crop Protection 63:68-75.

Tan J, Bednarek P, Liu J, Schneider B, Svatos A, Hahlbrock K (2004) Universally occurring phenylpropanoid and species-specific indolic metabolites in infected and uninfected *Arabidopsis thaliana* roots and leaves. Phytochem 65(6):691-9.

Tanaka K, Choi J, Cao Y, Stacey G (2014) Extracellular ATP acts as a damage-associated molecular pattern (DAMP) signal in plants. Front Plant Sci 5:446.

Tatsuki M, Mori H (1999) Rapid and transient expression of 1-aminocyclopropane-1-carboxylate synthase isogenes by touch and wound stimuli in tomato. Plant Cell Physiol 40:709–715.

Telewski FW (1989) Structure and function of flexure wood in *Abies fraseri*. Tree Physiol 5:113–121.

Telewski FW (2006) A unified hypothesis of mechanoperception in plants. Amer J Bot 93:1466–76.

Telewski FW, Jaffe MJ (1986*a*) Thigmomorphogenesis: Field and laboratory studies of *Abies fraseri* in response to wind or mechanical perturbation. Physiol Plant 66:211–218.

Telewski FW, Jaffe MJ (1986*b*) Thigmomorphogenesis: anatomical, morphological and mechanical analysis of genetically different sibs of *Pinus taeda* in response to mechanical perturbation. Physiol Plant 66:219–226.

Telewski FW, Pruyn ML (1998) Thigmomorphogenesis: a dose response to flexing in *Ulmus americana* seedlings. Tree Physiol 18:65–68.

Temmei Y, Uchida S, Hoshino D, Kanzawa N, Kuwahara M, et al. (2005) Water channel activities of *Mimosa pudica* plasma membrane intrinsic proteins are regulated by direct interaction and phosphorylation. FEBS Lett 579:4417–4422.

Thulke OU, Conrath U (1998) Salicylic acid has a dual role in the activation of defense-related genes in parsley. Plant J 14:35–42.

Tintor N, Ross A, Kanehara K, Yamada K, Fan L, et al. (2013) Layered pattern receptor signaling via ethylene and endogenous elicitor peptides during *Arabidopsis* immunity to bacterial infection. Proc Natl Acad Sci USA 110:6211–6216.

Tretner C, Huth U, Hause B (2008) Mechanostimulation of *Medicago truncatula* leads to enhanced levels of jasmonic acid. J Exp Bot 59:2847–2856.

Uppalapati SR, Marek SM, Lee HK, Nakashima J, Tang Y, et al. (2009) Global gene expression profiling during *Medicago truncatula-Phymatotrichopsis omnivora* interaction reveals a role for jasmonic acid, ethylene, and the flavonoid pathway in disease development. Mol Plant Microbe Interact 22:7–17.

Vallad GE, Goodman RB (2004) Systemic acquired resistance and induced systemic resistance. Crop Sci 44:1920-1934.

Van Dam NM, Baldwin IT (2001) Competition mediates costs of jasmonate-induced defences, nitrogen acquisition and transgenerational plasticity in *Nicotiana attenuata*. Funct Ecol 15:406–415.

Van der Meer JW, Joosten LA, Riksen N, Netea MG (2015) Trained immunity: a smart way to enhance innate immune defence. Mol Immunol 68(1):40–44.

Van Hulten M, Pelser M, van Loon LC, Pieterse CMJ, Ton J (2006) Costs and benefits of priming for defense in *Arabidopsis*. Proc Natl Acad Sci USA 103:5602–5607.

Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol 55:85–97.

Van Peer R, Neimann GJ, Schippers B (1991) Induced resistance and phytoalexin accumulation in biological control of *Fusarium* wilt of carnation by *Pseudomonas* sp. strain WCS417r. Phytopathology 81:728–734.

Van Peer R, Schippers B. (1992) Lipopolysaccharides of plant-growth promoting *Pseudomonas* sp. strain WCS417r induce resistance in carnation to *Fusarium* wilt. Netherlands Journal of Plant Pathol 98:129.

VanEtten HD, Matthews DE, Matthews PS (1989) Phytoalexin detoxification: importance for pathogenicity and practical implications. Annu Rev Phytopathol 27:143–164.

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W (2010) Lignin biosynthesis and structure. Plant Physiol 153(3): 895-905.

Visnovitz T, Világi I, Varró P, Kristóf Z (2007) Mechanoreceptor Cells on the Tertiary Pulvini of *Mimosa pudica* L. Plant Signal Behav 2:462–466.

Vogt T (2010) Phenylpropanoid biosynthesis. Mol Plant 3:2–20.

Volkov AG, Adesina T, Markin VS, Jovanov E (2008) Kinetics and mechanism of *Dionaea muscipula* trap closing. Plant Physiol 146:694–702.

Volkov AG, Foster JC, Ashby TA, Walker RK, Johnson JA, Markin VS (2010) *Mimosa pudica*: Electrical and mechanical stimulation of plant movements. Plant Cell Environ 33:163–173.

Wagner WL, Herbst DR, Sohmer SH (1999) Manual of the flowering plants in Hawaii. Vol 1. University of Hawaii Press, Bishop Museum, Honolulu, Hawaii.

Wan J, Zhang X-C, Neece D, Ramonell KM, Clough S, et al. (2008) A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. Plant Cell 20:471–481.

Wanner LA, Li G, Ware D, Somssich IE, Davis KR (1995) The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. Plant Mol Biol 27:327-338.

Wei G, Kloepper JW, Tuzun S (1991) Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. Phytopathology 81:1508-1512.

Wei X, Wang L, Zhang Y, Qi X, Wang X, et al. (2014) Development of simple sequence repeat (SSR) markers of sesame (*Sesamum indicum*) from a genome survey. Molecules 19:5150-62.

Whitehead FH, Luti R (1962) Experimental studies on the effect of wind on plant growth and anatomy. New Phytol 61:56–58.

Whitesell CD (1990) Silvical characteristics of Acacia koa Gray. *In*: Burns HRM, Honkala BH (eds) Agriculture Handbook 654. USDA Forest Service, Washington, D.C. pp 17–28.

Wiese J, Kranz T, Schubert S. (2004) Induction of pathogen resistance in barley by abiotic stress. Plant Biol 6:529-536.

Wilkins T, Smart LB. (1996) Isolation of RNA from Plant Tissue *In*: PA Krieg (ed) A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis. John Wiley & Sons, Inc, Hoboken, NJ. p. 21-42.

Wilkinson KM, Elevitch CR (2003) Growing koa, a Hawaiian legacy tree. Permanent Agriculture Resources, Holualoa, HI.

Wilkinson KM, Elevitch CR (2005) Propagation protocol for koa (*Acacia koa* Gray) Native Plants J 6:228-232.

Wilton IA, Bond BH, Dudley NS (2015) Review of wood properties *of Acacia koa* A. Gray (koa). Eur J Wood Wood Prod 73(1):121–126.

Wong MML, Cannon CH, Wickneswari R (2011) Identification of lignin genes and regulatory sequences involved in secondary cell wall formation in *Acacia auriculiformis* and *Acacia mangium* via *de novo* transcriptome sequencing. BMC Genomics 12:342.

Wu S, Zhu Z, Fu L, Niu B, Li W (2011) WebMGA: a customizable web server for fast metagenomic sequence analysis. BMC Genomics 12:444.

Wu Y, Yi G, Peng X, Huang B, Liu E, Zhang J (2013) Systemic acquired resistance in Cavendish banana induced by infection with an incompatible strain of *Fusarium oxysporum* f. sp. *cubense*. J Plant Phys 170(11):1039-46.

Wurzinger B, Mair A, Pfister B, Teige M (2011) Cross-talk of calcium-dependent protein kinase and MAP kinase signaling. Plant Signal Behav 6:8–12.

Xiong L and Yang Y (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid–inducible mitogen-activated protein kinase. Plant Cell15(3):745–759.

Yadeta KA, Valkenburg DJ, Hanemian M, Marco Y, Thomma BP (2014) The *Brassicaceae*-specific EWR1 gene provides resistance to vascular wilt pathogens. PLoS One 9(2):e88230.

Yamaguchi Y, Huffaker A (2011) Endogenous peptide elicitors in higher plants. Curr Opin Plant Biol 14:351–357.

Yanagida JF, Friday JB, Illukpitiya P, Mamiit RJ, Edwards Q (2004) Economic value of Hawaii's forest industry in (2001) Economic Issue 7. College of Tropical Agriculture and Human Resources. University of Hawai'i at Mānoa.

Yu P, Hegeman AD, Cohen JD (2014) A facile means for the identification of indolic compounds from plant tissues. Plant J 79(6):1065-75.

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, et al. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium-mediated* transformation. Cell 125:749–760.

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, et al. (2004). Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature 428: 764–767.

Zubieta C, Kota P, Ferrer JL, Dixon RA, Noel JP (2002) Structural basis for the modulation of lignin monomer methylation by caffeic acid/5- hydroxyferulic acid 3/5-O-methyltransferase. Plant Cell 14:1265-1277.